PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	Group Art Unit: 1634
Devin DRESSMAN et al.	)	
	)	Examiner: Woolwine, Samuel C.
	)	
Serial No. 10/562,840.	)	Confirmation No.: 6445
	)	
Filed: June 22, 2006	)	Atty. Dkt. No. 001107.00581

For: ***METHOD AND COMPOSITIONS FOR DETECTION AND ENUMERATION OF GENETIC VARIATIONS***

DECLARATION UNDER RULE 131

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

We, Devin Dressman, Hai Yan, Bert Vogelstein, and Kenneth W. Kinzler, declare:

1. We contributed a manuscript to the Proceedings of the National Academy of Sciences on June 6, 2003. Exhibit A, line 7.

2. The manuscript was accepted for publication in the same form as submitted. Exhibit A, line 7, which indicates no editing or resubmission.

3. The manuscript was published on-line on July 11, 2003, and appeared in print in volume 100, at pages 8817-8822. Exhibit B.

4. We began preparing the manuscript prior to June 6, 2003, the date on which we submitted the manuscript to the Proceedings of the National Academy of Sciences. An earlier version of the manuscript was saved prior to June 6, 2003. Exhibit C.

5. Prior to June 6, 2003, the date on which we submitted the manuscript to the Proceedings of the National Academy of Sciences, we performed or supervised the experiments and collected and analyzed the data described in the manuscript in the United States (Exhibit A). Specifically, as recited in independent claim 35, we formed microemulsions comprising DNA molecules. (Exhibit A, page 8817, last paragraph; page 8818, last paragraph, and Fig. 2.) We amplified analyte DNA molecules in the microemulsions in the presence of reagent beads. (Exhibit A, page 8818, second full paragraph, and page 8819, first full paragraph.) Product beads were formed which were bound to a plurality of copies of one species of analyte DNA molecule. (Exhibit A, page 8819, last full paragraph, and page 8820, fifth full paragraph and Fig. 3.) We separated product beads from analyte DNA molecules which were not bound to product beads. (Exhibit A, page 8818, third full paragraph.) We determined a sequence feature of the analyte DNA molecule bound to the product beads. (Exhibit A, Figs. 3, 4, 5.)

6. Prior to June 6, 2003, as recited in claim 36, we isolated product beads which were bound to a plurality of copies of a first species of analyte DNA molecule from product beads which were bound to a plurality of copies of a second species of analyte DNA molecule. (Exhibit A, page 8818, column 2, first full paragraph; page 8821-8822, spanning paragraph.)

7. Prior to June 6, 2003, as recited in claim 37, we isolated product beads using fluorescence activated cell sorting. (Exhibit A, page 8818, column 2, first full paragraph; page 8820, column 1, first full paragraph; page 8821-8822, spanning paragraph; Figs. 3, 4, and 5.)

8. Prior to June 6, 2003, as recited in claim 38, we recovered the first species of analyte DNA molecule from product beads. (Exhibit A, page 8821-8822, spanning paragraph; Fig. 5.)

9. Prior to June 6, 2003, as recited in claim 39, we amplified the first species of analyte DNA molecules from the isolated bead products. (Exhibit A, page 8821-8822, spanning paragraph; Fig. 5.)

10. Prior to June 6, 2003, as recited in claim 40, we determined the sequence of the first species analyte DNA molecule. (Exhibit A, page 8821-8822, spanning paragraph; Fig. 5.)

11. Prior to June 6, 2003, as recited in claim 41, we demonstrated that the amplifying step can convert less than 10% of the reagent beads present in the microemulsions into product beads. (Exhibit A, page 8820, paragraph spanning column 1 and 2.)

12. Prior to June 6, 2003, as recited in claim 42, prior to the step of separating, we used one or more detergents to break the microemulsions. (Exhibit A, page 8818, column 1, third full paragraph; page 8819, column 2, first full paragraph.)

13. Prior to June 6, 2003, as recited in claim 43, we determined a sequence feature of a species of the analyte DNA molecule using hybridization to oligonucleotide probes which are differentially labeled. (Exhibit A, page 8818, paragraph spanning column 1 and 2; page 8819-8820, spanning paragraph.)

14. Prior to June 6, 2003, as recited claimed in claim 44, we determined the relative or absolute amounts of product beads comprising one or more sequence features. (Exhibit A, page 8820, paragraph spanning column I and 2; page 8821, paragraph spanning column 1 and 2; Figs. 3, 4.)

15. Prior to June 6, 2003, as recited in claim 45, we used flow cytometry to determine the relative or absolute amounts of product beads comprising one or more sequence features. (Exhibit A, page 8820, paragraph spanning column 1 and 2; page 8821, paragraph spanning column 1 and 2; Figs. 3, 4.)

16. Prior to June 6, 2003, as recited claimed in claim 46, we amplified DNA analyte molecules using additional copies of the primer which are not bound to the reagent bead. (Exhibit A, page 8819, paragraph spanning columns 1 and 2.)

17. Prior to June 6, 2003, as recited in claim 47, we used analyte DNA that was genomic DNA. (Exhibit A, page 8818, column 2, second full paragraph; page 8821, column 1, first full paragraph; Fig. 4.)

18. Prior to June 6, 2003, as recited in claim 48, we used analyte DNA that was cDNA. (Exhibit A, page 8818, column 2, second full paragraph; Fig. 4.)

19. Prior to June 6, 2003, as recited in claim 49, we used analyte DNA molecules that were PCR products made from genomic DNA. (Exhibit A, page 8818, column 2, second full paragraph; page 8820-8821, spanning paragraph; Fig. 3.)

20. Prior to June 6, 2003, as recited in claim 50, we used analyte DNA molecules that were PCR products made from cDNA. (Exhibit A, page 8818, column 2, second full paragraph; page 8821, paragraph spanning column 1 and 2; Fig. 4.)

21. Prior to June 6, 2003, as recited in claim 51, we used analyte DNA molecules derived from a single individual. (Exhibit A, page 8820, paragraph spanning column 1 and 2; page 8821, paragraph spanning column 1 and 2; Figs. 3, 4.)

22. Prior to June 6, 2003, as recited in claim 52, we used analyte DNA molecules derived from a population of individuals. (Exhibit A, page 8817, abstract; page 8822, second column, second full paragraph.)

23. Prior to June 6, 2003, as recited in claim 53, using magnetic beads. (Exhibit A, page 8818, column 1, third full paragraph; page 8819, column 2, first full paragraph.)

24. Prior to June 6, 2003, as recited in claim 54, we determined a sequence feature by performing extension of a primer with one or more labeled deoxyribonucleotides. (Exhibit A, page 8818, column 2, second full paragraph.)

25. Prior to June 6, 2003, as recited in independent claim 59, we formed microemulsions comprising DNA molecules. (Exhibit A, page 8817, last paragraph; page 8818, last paragraph, and Fig. 2.). We amplified analyte DNA molecules in microemulsions in the presence of reagent beads and formed product beads bound to a plurality of copies of one species of analyte DNA molecule. (Exhibit A, page 8817, last paragraph; page 8818, last paragraph, Fig. 2; page 8818, second full paragraph, and page 8819, first full paragraph; page 8819, last full paragraph; page 8820, fifth full paragraph; and Fig. 3.) We separated product beads from analyte DNA molecules which were not bound to product beads. (Exhibit A, page 8818, third full paragraph.) We isolated product beads which were bound to a plurality of copies of a first species of analyte DNA molecule from product beads which were bound to a plurality of copies of a second species of analyte DNA molecule. (Exhibit A, page 8818, column 2, first full paragraph; page 8821-8822, spanning paragraph; Fig. 3, 4, and 5.)

26. Prior to June 6, 2003, as recited in claim 60, we isolated product beads using fluorescence activated cell sorting. (Exhibit A, page 8818, column 2, first full paragraph; page 8820, column 1, first full paragraph; page 8821-8822, spanning paragraph; Figs. 3, 4, and 5.)

27. Prior to June 6, 2003, as recited in claim 61, we recovered the first species of analyte DNA molecule from product beads. (Exhibit A, page 8821-8822, spanning paragraph; Fig. 5.)

28. Prior to June 6, 2003, as recited in claim 62, we amplified the first species of analyte DNA molecules from the isolated bead products. (Exhibit A, page 8821-8822, spanning paragraph; Fig. 5.)

29. Prior to June 6, 2003, as recited in claim 63, we determined the sequence of the first species analyte DNA molecule. (Exhibit A, page 8821-8822, spanning paragraph; Fig. 5.)

30. Prior to June 6, 2003, we collected data querying the *calpain-10* locus. We made and analyzed cDNA in emulsions that contained about 50 pg of PCR products synthesized from DNA of lymphoblastoid cells to query the *calpain-10* locus. See Exhibits D, E, F, and G, which represent our data collection and analysis with dates redacted. The green and red regions correspond to the A and G alleles for *calpain-10*.

31. Prior to June 6, 2003, we collected data querying the *MID42* locus. We made mixtures of PCR products containing 0-4 % *L* alleles of *MID42*, and amplified them in microemulsions and analyzed them. Flow cytometry was used to determine the fraction of singlet beads that were "red." The proportion of singlet beads that hybridized to at least one of the probes varied from 3.2 % to 4.3 %. Beads were sorted with the FACSVantage SE instrument (BD Biosciences), and individual red or green beads were used as templates for conventional PCR. Exhibits H-K, which represent our data collection and analysis with dates redacted, show data with different mixtures.

32. Prior to June 6, 2003, we collected data querying the *MID42* locus. We made and analyzed genomic DNA in emulsions that contained 10 and 1 ug of human

genomic DNA in the microemulsions to query the *MID42* locus. See Exhibits L and M, which represent our data collection and analysis with dates redacted. The green and red regions correspond to the *L* and *S* alleles for *MID42*.

33. We hereby declare that all statements made herein of our knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date	Devin Dressman, Ph.D.
Date	Kenneth Kinzler, Ph.D.
3-19-09	
Date	Hai Yan, M.D., Ph.D.
Date	Bert Vogelstein, M.D.

genomic DNA in the microemulsions to query the *MID42* locus. See Exhibits L and M, which represent our data collection and analysis with dates redacted. The green and red regions correspond to the *L* and *S* alleles for *MID42*.

33. We hereby declare that all statements made herein of our knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/19/09 _____ Devin Dressman, Ph.D.
Date

3/19/09 _____ Kenneth Kinzler, Ph.D.
Date

_____ Hai Yan, M.D., Ph.D.
Date

3.19.09 _____ Bert Vogelstein, M.D.
Date

Exhibit A

Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations

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Contributed by Bert Vogelstein, June 6, 2003

Many areas of biomedical research depend on the analysis of uncommon variations in individual genes or transcripts. Here we describe a method that can quantify such variation at a scale and ease heretofore unattainable. Each DNA molecule in a collection of such molecules is converted into a single magnetic particle to which thousands of copies of DNA identical in sequence to the original are bound. This population of beads then corresponds to a one-to-one representation of the starting DNA molecules. Variation within the original population of DNA molecules can then be simply assessed by counting fluorescently labeled particles via flow cytometry. This approach is called BEAMing on the basis of four of its principal components (beads, emulsion, amplification, and magnetics). Millions of individual DNA molecules can be assessed in this fashion with standard laboratory equipment. Moreover, specific variants can be isolated by flow sorting and used for further experimentation. BEAMing can be used for the identification and quantification of rare mutations as well as to study variations in gene sequences or transcripts in specific populations or tissues.

The study of DNA sequence variation is important for many areas of research. The study of germ-line variations is essential for assessing the role of inheritance in normal and abnormal physiologic states (1). Other variations, developed somatically, are responsible for neoplasia (2). The identification of such mutations in urine, sputum, and stool can therefore be used for the detection of presymptomatic cancers (3–5). Similarly, the detection of somatic mutations in lymph nodes, blood, or bone marrow can provide data about the stage of disease, prognosis, and appropriateness of various therapies (5). Somatic mutations in nonneoplastic cells also occur and seem to accumulate as humans age or are exposed to environmental hazards (6). Such mutations occur in only a small fraction of the cells in a tissue, thereby complicating their analysis.

Central to the investigation of many of these issues is the detection and quantification of sequence variants within a population of DNA molecules. The number of molecules in each such collection is finite and therefore countable. Consider, for example, a collection of red and green balls. Counting these balls is simple in principle but subject to basic probability theory. If there is only one red ball for every 500 green balls, then it is necessary to count several thousand balls to get an accurate estimate of the proportion of red balls. If it is difficult to count enough balls to make a reliable estimate, one can elute the paint off all the balls and measure the color of the resultant paint mix.

In analogous fashion, small numbers of DNA molecules that vary by subtle changes (single base-pair substitutions or small deletions or insertions) can be counted directly by amplifying individual DNA molecules (single-molecule PCR) (7–12, 29). Such digital techniques have been shown to be extremely useful for measuring variation in genes or their transcripts, but digital technologies have been limited thus far to counting tens to thousands of molecules in the wells of microtiter plates, on microscope slides, or after electrophoresis of individual PCR products. Analog techniques, analogous to the elution of paint from the balls described above, are generally easier to implement and can assess millions of molecules simultaneously (e.g. ref. 13). However, their accuracy and sensitivity are limited by instrumental and experimental noise.

In this article we describe a digital technology called BEAMing (on the basis of four of its principal components: beads, emulsion, amplification, and magnetics), which has the power to assess millions of molecules and can be generally applied to the study of genetic variation. The technology involves conversion of single DNA molecules to single magnetic beads, each containing thousands of copies of the sequence of the original DNA molecule. The number of variant DNA molecules in the population then can be assessed by staining the beads with fluorescent probes and counting them by using flow cytometry. Beads representing specific variants can be recovered through flow sorting and used for subsequent confirmation and experimentation.

Materials and Methods

Step 1: Coupling Oligonucleotides to Beads. Superparamagnetic beads of 1.05 ± 0.1 μm in diameter, covalently bound to streptavidin, were purchased from Dynal Biotech (no. 650.01, Lake Success, NY). Beads were washed once with 1× PCR buffer (50 mM KCl/20 mM Tris·HCl, pH 8.4) and then suspended in bind-and-wash buffer (5 mM Tris·HCl/0.5 mM EDTA/1.0 M NaCl, pH 7.5). Beads were incubated in bind-and-wash buffer for 30 min at room temperature in the presence of 10 μM oligonucleotides (Table 1, which is published as supporting information on the PNAS web site, www.pnas.org). These oligonucleotides were modified with a dual biotin group at the 5′ end, with the biotin groups separated by a six-carbon linker (Integrated DNA Technologies, Coralville, IA). After binding, the beads were washed three times with 1× PCR buffer to thoroughly remove unbound oligonucleotides.

Step 2: Preparing Microemulsions. Microemulsions for PCR were prepared by slight modifications of described methods (14, 15). The oil phase was composed of 4.5% Span 80 (no. S6760, Sigma), 0.40% Tween 80 (no. S-8074, Sigma), and 0.05% Triton X-100 (no. T9284, Sigma) in mineral oil (no. M-3516, Sigma). The oil phase was freshly prepared each day. The aqueous phase consisted of 67 mM Tris·HCl (pH 8.8), 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 0.05 μM forward primer, 25 μM

Abbreviation: BEAM, beads, emulsion, amplification, and magnetics.

*Under an agreement between Exact Sciences, Inc., and Johns Hopkins University (JHU), D.D., H.Y., G.T., K.W.K., and B.V. are entitled to a share of the royalties received by JHU on sales of products related to the use of digital PCR technologies. JHU also owns Exact Sciences, Inc., stock, which is subject to certain restrictions under JHU policy. The terms of these arrangements are being managed by JHU in accordance with its conflict-of-interest policies.

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reverse primer, 45 units of Platinum *Taq* (no. 10966-034, Invitrogen), various amounts of template DNA (see *Results*), and $\approx 10^8$ oligonucleotide-coupled beads in a total volume of 300 μl. The forward primer was an oligonucleotide with a sequence that was identical to the 3′ 20–22 nt of that described in step 1 and was not modified with biotin.

Water-in-oil microemulsions were prepared by dropwise addition of 200 μl of the aqueous phase to 400 μl of the oil phase previously placed in a 2-ml round-bottom cryogenic vial (no. 430661, Corning). The dropwise addition was performed over ≈1 min while the mixture was being stirred at 1,400 rpm with a magnetic microstir bar (no. 58948-353, VWR Scientific) on a VWR model 565 magnetic stirrer. After the addition of the aqueous phase, the mixture continued to be stirred for a total time of 30 min. Two emulsions were made at once by placing two tubes in a rack placed at the center of the magnetic stirrer.

Step 3: PCR Cycling. The emulsions were aliquoted into five wells of a 96-well PCR plate, each containing 100 μl. PCR was carried out under the following cycling conditions: 94°C for 2 min, 40 cycles of 94°C for 15 sec, 57°C for 30 sec, and 70°C for 30 sec. The PCR products analyzed in this study ranged from 189 to 239 bp.

Step 4: Magnetic Capture of Beads. After PCR cycling, the microemulsion from five wells of a PCR plate were pooled and broken by the addition of 800 μl of NX buffer (100 mM NaCl/1% Triton X-100, 10 mM Tris·HCl, pH 7.5, and 1 mM EDTA) in a 1.5-ml tube (no. 430909, Corning). After vortexing for ≈20 sec, the beads were pelleted by centrifugation in a microcentrifuge at 8,000 rpm (5,000 × *g*) for 90 sec. The top oil phase and all but ≈300 μl of the aqueous phase were removed from the tube, and 600 μl of NX buffer was added. After vortexing for 20 sec and centrifugation for 90 sec, the top oil phase and all but ≈300 μl of the aqueous phase were removed. The addition of 600 μl of NX buffer, vortexing, and centrifugation were repeated once more, and the top oil portion and all but ≈300 μl of the aqueous phase were removed. The tube then was placed on a magnet (MPC-S, Dynal), and the rest of the supernatant was pipetted off carefully. The beads were washed an additional three times with 1× PCR buffer by using magnetic separation rather than centrifugation and finally resuspended in 100 μl of 1× PCR buffer.

Step 5: Sequence Differentiation. Two oligonucleotide probes were used for each reaction. One was 5′-labeled with 6-carboxyfluorescein and was specific for one allele, whereas the second was 5′-labeled with biotin and was specific for the other allele. Probes were synthesized by Integrated DNA Technologies. The 30-μl hybridization reactions contained 10 μM of each probe and 5–25 million beads in 1× PCR buffer. Reactions were performed in PCR plates on a thermal cycler by heating to 94°C for 30 sec and then cooling to 75°C at a rate of 0.5°C/sec, cooling to 45°C at 0.2°C/sec, and finally cooled to 30°C at 1°C/sec. All subsequent steps were performed at room temperature. The reactions were transferred to a 96-well Costar plate (no. 3797, Corning) and placed on a 96-well magnet. Beads were collected magnetically by exposing them to the magnet for 2 min. The supernatant was removed and the beads washed three times with 1× PCR buffer by pipetting them and collecting for 2 min. They were finally resuspended in 100 μl of B-PCR buffer (1 mg/ml BSA in 1× PCR buffer). The beads then were incubated for 10 min in a total volume of 100 μl of B-PCR buffer containing 3 μg of Alexa-488 rabbit anti-fluorescein antibody (no. A-11090, Molecular Probes) and 3 μg of Nutravidin labeled with R-phycoerythrin (no. A-2660, Molecular Probes) in B-PCR buffer. The beads were washed three times and resuspended in B-PCR buffer as described above. They then were incubated for 10 min in a total volume of 100 μl of B-PCR buffer containing 6 μg of Alexa 488-conjugated chicken anti-rabbit antibody (no. A-21441, Molecular Probes) and 3 μg of biotinylated goat anti-avidin antibody (no. BA-0300, Vector Laboratories). The beads were washed three times and resuspended in B-PCR buffer as described above. They then were incubated for 10 min in a total volume of 100 μl of B-PCR buffer containing 3 μg of an Alexa 488-conjugated goat anti-chicken antibody (no. A-11039, Molecular Probes) and 3 μg of R-phycoerythrin-labeled streptavidin (no. S-866, Molecular Probes). This solution then was washed an additional three times with 1× PCR buffer and resuspended in 20 μl of 1× PCR buffer.

Step 6: Flow Cytometry. The bead suspension was diluted to a concentration of $\approx 10^6$ to 10^7 beads per ml in 10 mM Tris·HCl/1 mM EDTA (no. 351-010-131, Quality Biologicals, Gaithersburg, MD) and analyzed by using an LSR instrument (BD Biosciences, Franklin Lakes, NJ). The instrument was set up for standard two-color analysis by using an argon laser and optical filters that distinguished between the two fluorescent dyes. No spectral deconvolution was required because the major bead populations were well separated. In some cases, scanning was performed with FACScan or FACSCalibur instruments (BD Biosciences), yielding equivalent results. Sorting was carried out with a FACSVantage SE instrument (BD Biosciences). The flow-cytometry data were analyzed by using CELLQUEST software (BD Biosciences).

Template Preparation and Sequence Analyses. Human genomic DNA was purified with DNeasy (no. 69504, Qiagen, Valencia, CA). RNA was purified with Quickprep (no. 27-9255-01, Amersham Pharmacia Biosciences). Reverse transcription of RNA was performed by using Superscript II reverse transcriptase (no. 18064014, Invitrogen) according to manufacturer instructions. PCR with genomic DNA or reverse transcripts as templates was performed as described (7). PCR products to be used as templates for BEAMing or sequencing were purified with QIAquick (no. 28104, Qiagen). Sequencing reactions were performed by using BigDye v3.0 reagents (Applied Biosystems) and analyzed by capillary electrophoresis (Spectrumedix 9600, State College, PA).

Results

BEAMing consists of the six steps diagrammed in Fig. 1.

Step 1: Coupling Oligonucleotides to Beads. We used streptavidin beads because of the simplicity of coupling biotinylated oligonucleotides to them. Oligonucleotides with just a single 5′-biotin group were found to dissociate from the beads during temperature cycling, whereas oligonucleotides labeled with dual biotin groups at their 5′ end (separated by a six-carbon linker) were stable to cycling. As determined by fluoroscopic measurements of oligonucleotides doubly labeled with 6-carboxyfluorescein and biotin, $\approx 10^5$ oligonucleotide molecules were bound to each bead. We found that short oligonucleotides (20 bases) did not work as well for priming as longer ones (41 bp), perhaps because of steric hindrance at the bead surface. It is likely that amino-, sulfhydryl-, or carboxyl-modified oligonucleotides covalently coupled to beads modified with corresponding reactive groups could also function as bead-bound primers for BEAMing.

Step 2: Preparing Microemulsions. The size of the individual aqueous compartments were 5.4 ± 2.7 μm in diameter (Fig. 2). We estimated that an emulsion comprising 200 μl of aqueous solution and 400 μl of oil would contain $\approx 3 \times 10^9$ compartments with an average diameter of 5 μm. Approximately 10^8 beads were included in each emulsion such that only one in ≈30 compartments contained a bead. The optimal amount of template was experimentally determined to be $\approx 5 \times 10^8$ molecules, so that

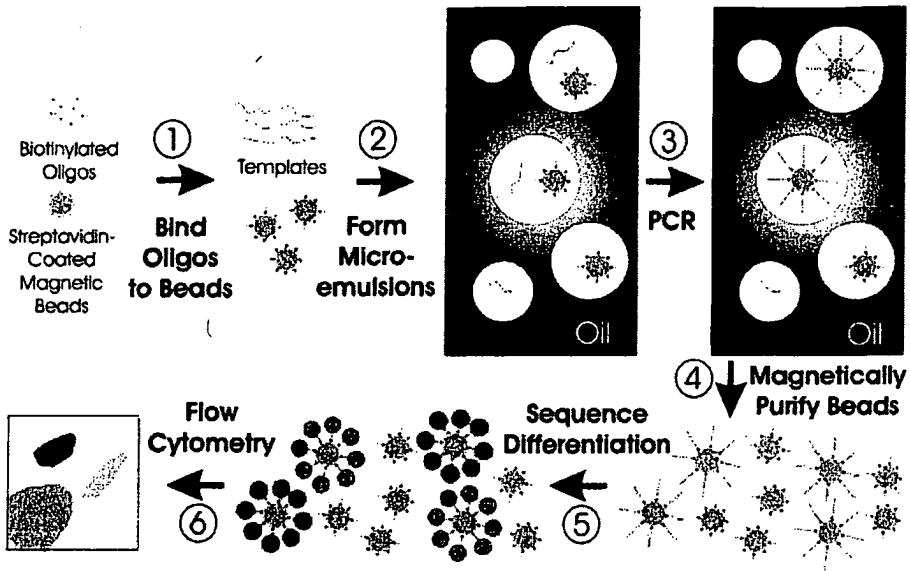

Fig. 1. Schematic of BEAMing. Step 1: Magnetic beads covalently coated with streptavidin are bound to biotinylated oligonucleotides (oligos). Step 2: An aqueous mix containing all the necessary components for PCR plus primer-bound beads and template DNA are stirred together with an oil/detergent mix to create microemulsions. The aqueous compartments (white circles in the gray oil layer) contain an average of less than one template molecule and less than one bead. Red and green templates represent two template molecules, the sequences of which differ by one or many nucleotides. Step 3: The microemulsions are temperature-cycled as in a conventional PCR. If a DNA template and a bead are present together in a single aqueous compartment, the bead-bound oligonucleotides act as primers for amplification. The straight red and green lines connected to the beads represent extension products from the two different kinds of templates. Step 4: The emulsions are broken, and the beads are purified with a magnet. Step 5: After denaturation, the beads are incubated with oligonucleotides that can distinguish between the sequences of the different kinds of templates. Fluorescently labeled antibodies then are used to label the bound hybridization probes, which renders the beads containing PCR product as red or green after appropriate laser excitation. Step 6: Flow cytometry is used to count the red and green beads.

one in approximately six compartments contained a template molecule.

Step 3: PCR Cycling. PCR priming by oligonucleotides coupled to beads was found to be very inefficient compared with the priming by the same oligonucleotides when free in solution. For this reason, a small amount of nonbiotinylated forward primer identical in sequence to the biotinylated oligonucleotide coupled to the beads was included in the reactions. This facilitated the first few rounds of amplification of the single template within each aqueous compartment. In the absence of additional primer, no detectable amplification on the beads was generated. Conversely, if too much additional primer was included, no amplification on the beads occurred because of competition with the primers in solution. An excess of the reverse primer was included in the aqueous compartment to maximize the probability that bead-bound oligonucleotides extended by polymerase would serve as templates for further amplification cycles.

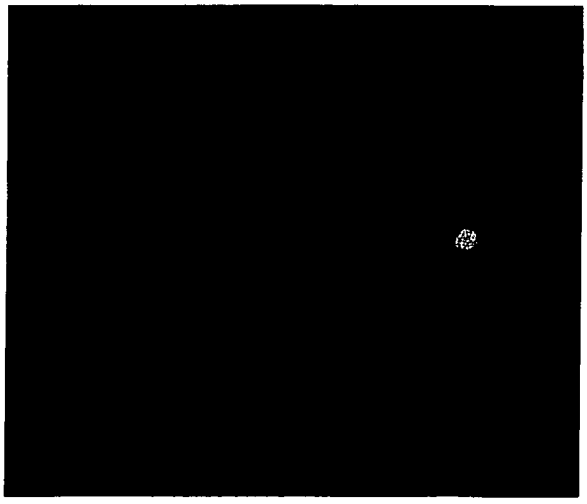

Fig. 2. Photograph of a typical microemulsion. Microemulsions were made as described in *Materials and Methods* with the exception that the aqueous compartments contained cascade blue-labeled dCTP and the beads were prelabeled by binding to oligonucleotides coupled to R-phycoerythrin (red) or Alexa 488 (green). One microliter of microemulsion was deposited in 1 μl of oil on a microscope slide before photography. Of the seven aqueous compartments visible in this picture, two contain beads. Note the heterogeneous size of the aqueous compartments (beads are 1.05 μm in diameter).

Step 4: Magnetic Capture of Beads. There are several ways to break water-in-oil emulsions including extraction with organics (14). We found that simply adding nonionic detergents produced phase separations without any detectable modification of the beads or DNA molecules bound to them. By measuring the amount of DNA that could be released from the beads after restriction endonuclease digestion, we estimate that >10,000 extended PCR products were present, on average, per bead.

Step 5: Sequence Differentiation. Most fluorescence-based methods for distinguishing alleles in homogeneous or two-phase assays can be used to assess allelic variation captured on beads. These methods include single nucleotide extension, allele-specific priming, or hybridization. We generally used hybridization of fluorescein-conjugated or biotin-conjugated oligonucleotides for discrimination. As shown in Fig. 1 and Table 1, these oligonucleotides had a stem–loop structure, with the middle of the loop containing the variant nucleotide(s). This design was based on studies of molecular beacons wherein a stem–loop structure was shown to improve allelic discrimination markedly

(16). The oligonucleotides we used differed from molecular beacons in that there was no need for a quenching group. Such quenching is required for homogeneous assays when unhybridized oligonucleotides cannot be removed from the reactions before assay but is not necessary for solid-phase assays such as those used with beads.

Step 6: Flow Cytometry. Optimum results in flow cytometry depend on high fluorescent signals on the beads. We generally enhanced the fluorescence emanating from the hybridization probes with secondary reagents. For example, Alexa 488-labeled antibodies were used to enhance the signals emanating from fluorescein-coupled oligonucleotide probes. Similarly, R-phycoerythrin-labeled streptavidin was used to generate a signal from biotin-labeled oligonucleotide probes. Flow cytometers equipped with two or three lasers and appropriate filters have the capacity to distinguish multiallelic loci and perform multiplex analysis of several genes simultaneously. The newest generation of flow cytometers can also analyze >70,000 events per sec. In addition to the analytical power of flow cytometry, fluorescence-activated cell sorter instruments can separate specific populations of beads for further analysis.

Characteristics of Microemulsions. Pilot experiments demonstrated that simply stirring the water-in-oil mixtures described in *Materials and Methods* produced very stable microemulsions of a size compatible with that of the beads. In the experiment shown in Fig. 2, the aqueous compartment contained a blue dye and 1-μm magnetic beads that were labeled by binding to fluorescently labeled oligonucleotides. The appearance of emulsions immediately after their formation is shown in Fig. 2. As expected, this appearance was unchanged after temperature cycling during PCR (15). Most aqueous compartments contained no beads, as expected from the calculations in step 2. Those compartments that did contain beads generally contained only one, although a fraction contained more, as expected from a Poisson distribution and nonuniform aqueous compartment sizes. "Heterozygous" beads containing PCR products representing both alleles are produced when two or more DNA template molecules are contained within a single aqueous compartment. Such heterozygotes can compromise the accuracy of the analyses under some circumstances (see *Discussion*).

Detection of Homozygotes and Heterozygotes. Fig. 3 shows typical results obtained with human DNA samples. The *MID42* marker used in this experiment was chosen from a collection of diallelic short insertion/deletion polymorphisms assembled by Weber *et al.* (17). These alleles are particularly simple to distinguish with hybridization probes because the two alleles at each locus differ by ≈4 bases. The probe for the longer (*L*) allele was labeled with fluorescein (green), and the probe for the shorter (*S*) allele was labeled with R-phycoerythrin (red).

Fig. 3*A* shows a plot of the side scatter vs. forward scatter of beads after BEAMing. In general, >75% of beads were dispersed as single particles, with the remainder aggregated in groups of two or more. Subsequent flow-cytometric analysis was confined to the singlet beads, gated as outlined in Fig. 3*A*.

Fig. 3 *B–D* show density plots of gated beads generated with various templates. In Fig. 3*B*, a template from an individual homozygous for the *L* allele was included in the emulsion. Two populations of beads were apparent. Ninety-eight percent of the beads contained no PCR product (black), and the remaining 2% fluoresced in the FL1 channel (colored green in Fig. 3). Fig. 3*C* represents the analysis of an individual homozygous for the *S* allele. Two populations of beads were again apparent; but this time the labeled population fluoresced in the FL2 channel (colored red in Fig. 3). Fig. 3*D* presents density plots from the analysis of an individual heterozygous at the *MID42* locus. Four

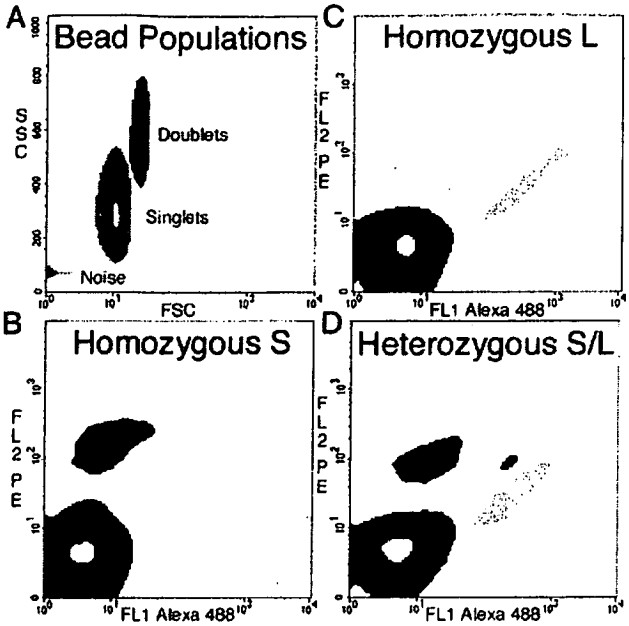

Fig. 3. Density plots of flow-cytometric data obtained from BEAMing. The locus queried in this experiment was *MID42*, and PCR products generated from genomic DNA were used as templates in the microemulsions. (*A*) Forward scatter (FSC) and side scatter (SSC) of all beads show that ~80% of the total beads are singlets, with most of the remaining beads aggregated as doublets. The "noise" is instrumental and is observed with blank samples containing no beads. The instrument output was gated such that only singlets were analyzed for fluorescence analysis. The patterns observed from an individual homozygous for the *L* allele (*A*), homozygous for the *S* allele (*B*), and heterozygous for *L* and *S* (*D*) are shown in *B–D*, respectively. The regions containing beads hybridizing to the *L* and *S* allele probes are labeled green and red, respectively. The region containing beads that did not hybridize to any probe is black, and the region containing beads that hybridized to both probes is blue. The blue beads arose from aqueous compartments in which both types of template molecules were present. The proportion of singlet beads that hybridized to at least one of the probes was 2.9%, 4.3%, and 20.3% in *B–D*, respectively. The forward-scatter and side-scatter plots in *A* represent the same beads analyzed in *D*. FL1, fluorescent channel 1; FL2, fluorescent channel 2; PE, R-phycoerythrin.

populations of beads are evident: the black region represents beads without any PCR product; the red region represents beads containing PCR products from the *S* allele; the green region represents beads containing PCR products from the *L* allele; and the blue region represents beads containing PCR products from both alleles. Beads containing PCR products from both alleles were derived from aqueous compartments that contained more than one template molecule. The number of such beads increased in a nonlinear fashion as more template molecules were added. At the extreme, when all aqueous compartments are saturated, virtually all beads will register as blue. Operationally, we found that the bead populations were most distinct when the number of beads containing any PCR product was <10% of the total beads analyzed.

PCR Products, Genomic DNA, or cDNA as Templates. The results shown in Fig. 3 were generated by using PCR products made from human genomic DNA samples. Because the ratio of the beads representing *L* alleles to those representing *S* alleles was 1.0 in this experiment, it was clear that the initial PCR did not preferentially amplify either allele. The use of PCR products rather than genomic DNA permitted large numbers of alleles to be amplified from even small quantities of starting DNA. In

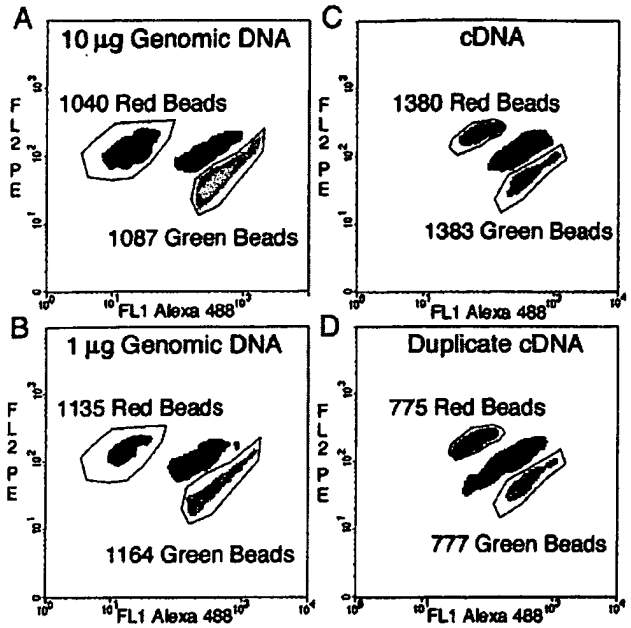

Fig. 4. Density plots of BEAMing with genomic DNA or RT-PCR products as templates. The data in *A* and *B* were generated by including 10 and 1 µg of human genomic DNA, respectively, in the microemulsions, querying the *MID42* locus. The data in *C* and *D* were generated by using emulsions that contained ~50 pg of PCR products synthesized from cDNA of lymphoblastoid cells, querying the *calpain-10* locus. The green and red regions correspond to the *L* and *S* alleles for *MID42* and to the *A* and *G* alleles for *calpain-10*. The number of beads in the outlined regions containing red or green beads is shown in each case. The proportion of singlet beads that hybridized to at least one of the probes was 1.2%, 0.6%, 6.8%, and 4.2% in *A–D*, respectively. The outlined regions used for counting in *A* and *B* were identical, as were those used for *C* and *D*. Beads that did not hybridize to any probe were gated out and therefore not evident in the graphs, and the region containing beads that hybridized to both probes is labeled blue. FL1, fluorescent channel 1; FL2, fluorescent channel 2; PE, R-phycoerythrin.

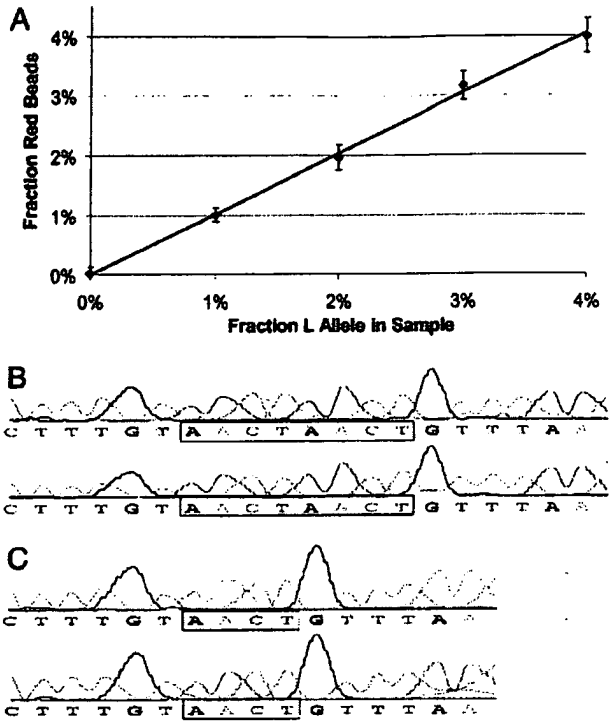

Fig. 5. Detection and validation of variants present in a minor fraction of the DNA population. (*A*) Mixtures of PCR products containing 0–4% *L* alleles of *MID42* were used for BEAMing. Flow cytometry such as that shown in Fig. 3 was used to determine the fraction of singlet beads that were red (*y* axis). The proportion of singlet beads that hybridized to at least one of the probes varied from 3.2% to 4.3%. (*B* and *C*) Beads were sorted with the FACSVantage SE instrument, and individual red or green beads were used as templates for conventional PCR by using the forward and reverse primers listed in Table 1. Red beads generated only the *S* allele sequence, whereas green beads generated only the *L* allele sequence.

general, 10–100 pg of PCR products 200 bp in size were found to be optimal for BEAMing, producing PCR-mediated extension of primers on ≈1–10% of labeled beads.

In some situations it might be useful to use genomic DNA rather than PCR products as templates for BEAMing. The data in Fig. 4 *A* and *B* show flow-cytometric data from an experiment wherein 10 or 1 µg of human genomic DNA was used as template for BEAMing at the *MID42* locus. Patterns very similar to those shown in Fig. 3 were observed, although fewer beads were labeled than when PCR products were used as templates.

BEAMing could also be used to analyze variations in expression from the two alleles of a heterozygous individual. Heritable variations in the expression from individual alleles of the same gene have been shown to occur often in humans (18) and mice (19) and can have significant phenotypic effects (20). The results shown in Fig. 4 *C* and *D* show that PCR products made from reverse-transcribed mRNA can be used for BEAMing. In this case, *calpain-10* transcripts differing by a single nucleotide polymorphism were analyzed. For single nucleotide polymorphisms such as these, probes that incorporated an extra mismatched nucleotide adjacent to the polymorphic nucleotide (see Table 1) can enhance the distinction between alleles (21, 22). The results from two independent emulsions made with aliquots of the same RT-PCR product are shown to illustrate reproducibility. Although the number of beads that functioned as templates in BEAMing varied up to 3-fold among experiments with identical templates, the proportion of beads representing the two alleles was reproducible (1,383 *A* allele beads to 1,380 *G* allele beads in Fig. 4*C* and 777 *A* allele beads to 775 *G* allele beads in Fig. 4*D*, respectively).

Analysis of Minor Variants in a DNA Population. The analysis of uncommon variations is ideally suited for analysis via BEAMing because of the large number of molecules that can be analyzed independently while retaining a high signal-to-noise ratio. Fig. 5*A* shows representative data from templates representing 1–4% of the *L* allele of *MID42*. The linearity of these measurements, with a correlation coefficient of 0.99, demonstrates the utility of this approach for such applications. We also applied this analysis to the detection of *KRAS* and could easily observe 0.1% mutants when spiked into a population of wild-type molecules (data not shown).

The rare beads representing the mutant alleles could not only be quantified but also purified for subsequent analysis. As a demonstration, samples of the beads enumerated in Fig. 5*A* were assessed by using a flow cytometer equipped with sorting capabilities. Beads were sorted and individual beads were used as templates for conventional PCR by using the same primers used for BEAMing. Because each bead contains thousands of bound template molecules, single beads were expected to generate robust PCR products (23), and this was confirmed experimentally. These PCR products then were subjected to sequenc-

ing. As shown in Fig. 5 *B* and *C*, green and red beads generated PCR products exclusively of the *L* and *S* types, respectively.

Discussion

The results described above show that BEAMing provides a reliable and sensitive assay for measuring variations in genes and transcripts. It requires no instrumentation other than a magnetic stirrer, a temperature cycler, and a flow cytometer, all of which are widely available. There are several other advantages of this approach. First, the sensitivity can be increased to meet the specifications of the experiment simply by analyzing more beads. Such sensitivity is limited only by the error rate of the polymerases used for amplification. Second, BEAMing data can be used not only to demonstrate that a variant is present in a particular population of DNA molecules but also quantifies the fraction of variant DNA molecules in that population (Fig. 5*A*). Such quantification is not possible with techniques that destroy or ignore the wild-type molecules as part of the assay, such as those that use allele-specific priming or endonuclease digestion during PCR. Third, the beads containing variant alleles can be purified easily through flow sorting and used for subsequent experimentation. Such recovery is difficult with digital techniques that count molecules deposited on microscope slides. Finally, the BEAMing approach, in principle, is automatable.

Several modifications of the basic principles described here can be envisioned that will simplify the technology further or widen its applications. For example, microemulsions were made by stirring water/oil/detergent mixes. The sizes of the resultant aqueous compartments were somewhat heterogeneous, as illustrated in Fig. 2. A relatively large number of beads containing PCR products of both alleles are obtained from large compartments because they are more likely to contain more than one template molecule than smaller compartments. Although this is not a problem for the analysis of uncommon variants, it does pose a problem when the variant to be analyzed is present in a substantial fraction of the DNA molecules. For example, it is easy to distinguish a population containing 2% of allele *A* and 98% of allele *B* from one that contains 0% of allele *A* (Fig. 5*A*). But it is more difficult to distinguish a population that contains 48% of allele *A* and 52% of allele B from a population that contains 50% of allele *A*; the large number of heterozygote beads formed in the latter analysis diffuses the boundaries of the pure red and green channels. This limit to accuracy theoretically can be overcome through the preparation of more uniformly sized aqueous compartments, perhaps through sonication or pressure-driven emulsifiers.

Although flow cytometry requires only seconds to minutes per sample, multiple parallel analyses could facilitate throughput. Novel particle-counting designs may prove useful for this purpose (24, 25). Another way to increase throughput would be to physically separate the beads that contained PCR products before flow cytometry, which could be accomplished with antibodies to modified nucleotides incorporated into the PCR product during BEAMing.

Although we focused on issues related to human variation, BEAMing technology obviously could be applied to genes or transcripts of any organism or population of organisms. It could also be used to quantify epigenetic alterations such as methylation if DNA was first treated with bisulfite to convert methylated cytosine residues to thymidine. Beads generated from random fragments of whole genomes (26) rather than from individual genes as described above could be used to identify gene segments that bind to specific DNA-binding proteins (27). If the beads made by BEAMing were used in compartmentalized *in vitro* transcription-translation reactions, variant proteins would be bound to beads containing the corresponding variant DNA sequences (23), which could allow facile flow-cytometric evaluation of rare mutations by using antibodies that distinguish between wild-type and mutant gene products (28).

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Exhibit B

1: Proc Natl Acad Sci U S A. 2003 Jul 22;100(15):8817-22. Epub 2003 Jul 11.

Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations.

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Many areas of biomedical research depend on the analysis of uncommon variations in individual genes or transcripts. Here we describe a method that can quantify such variation at a scale and ease heretofore unattainable. Each DNA molecule in a collection of such molecules is converted into a single magnetic particle to which thousands of copies of DNA identical in sequence to the original are bound. This population of beads then corresponds to a one-to-one representation of the starting DNA molecules. Variation within the original population of DNA molecules can then be simply assessed by counting fluorescently labeled particles via flow cytometry. This approach is called BEAMing on the basis of four of its principal components (beads, emulsion, amplification, and magnetics). Millions of individual DNA molecules can be assessed in this fashion with standard laboratory equipment. Moreover, specific variants can be isolated by flow sorting and used for further experimentation. BEAMing can be used for the identification and quantification of rare mutations as well as to study variations in gene sequences or transcripts in specific populations or tissues.

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Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations

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Many areas of biomedical research depend on the analysis of uncommon variations in individual genes or transcripts. We here describe a method that can quantify such variation at a scale and ease heretofore unattainable. Each DNA molecule in a collection of such molecules is converted into a single magnetic particle to which thousands of copies of DNA identical in sequence to the original are covalently bound. This population of beads then corresponds to a one-to-one representation of the starting DNA molecules. Variation within the original population of DNA molecules can then be simply assessed by counting fluorescently-labeled particles via flow cytometry. This approach is called "BEAM"ing on the basis of four of its principal components (beads, emulsion, amplification, and magnetics). Millions of individual DNA molecules can be assessed in this fashion with standard laboratory equipment. Moreover, specific variants can be isolated by flow sorting and employed for further experimentation. BEAMing can be used for the identification and quantification of rare mutations as well as to study variations in gene sequences or transcripts in specific populations or tissues.

The study of DNA sequence variation is essential for many areas of research. Germ-line variations lie at the heart of methods to assess the role of inheritance in normal and abnormal physiologic states (1). Other variations, developed somatically, are responsible for neoplasia (2). The identification of such mutations in urine, sputum, and stool can thereby be used for the detection of presymptomatic cancers (3-5). Similarly, the detection of somatic mutations in lymph nodes, blood, or bone marrow can provide data about the stage of disease, prognosis, and appropriateness of various therapies (5). Somatic mutations in non-neoplastic cells also occur and appear to accumulate as humans age or are exposed to environmental hazards (6). Such mutations occur in only a small fraction of the cells in a tissue, thereby complicating their analysis.

Central to the investigation of many of these issues is the detection and quantification of sequence variants within a population of DNA molecules. The number of molecules in

each such collection is finite and therefore "countable". Consider, for example, a collection of red and green balls. Counting these balls is simple in principle but subject to basic probability theory. If there is only one red ball for every 500 green balls, then it is necessary to count several thousand balls to get an accurate estimate of the proportion of red balls. If it is difficult to count enough balls to make a reliable estimate, one can elute the paint off all the balls and measure the color of the resultant paint mix.

In analogous fashion, small numbers of DNA molecules that vary by subtle changes (single base pair substitution or small deletion or insertion) can be directly counted by amplifying individual DNA molecules (single molecule PCR) (7-12). Such "digital" techniques have been shown to be extremely useful for measuring variation in genes or their transcripts. But digital technologies have so far been limited to counting tens to thousands of molecules, either in the wells of microtiter plates, on microscope slides, or after electrophoresis of individual PCR products. "Analog" techniques, analogous to the elution of paint from the balls described above, are generally easier to implement (13). However, their accuracy and sensitivity is limited by instrumental and experimental noise.

In this communication, we describe a digital technology, called BEAMing, that can be generally applied to the study of genetic variation. The technology involves conversion of single DNA molecules to single magnetic beads each containing thousands of copies of the sequence of the original DNA molecule. The number of variant DNA molecules in the population can then be assessed by staining the beads with fluorescent probes and counting them using flow cytometry. Beads representing specific variants can be recovered through flow sorting and used for subsequent confirmation and experimentation.

Materials and Methods

Step 1 - Coupling oligonucleotides to beads.

Superparamagnetic beads of 1.05 +/- 0.1 um in diameter, covalently bound to streptavidin, were purchased from Dynal Biotech, Inc. (product # 650.01, Lake Success, NY). Beads were washed with 1x PCR buffer (#53286, Invitrogen, Carlsbad, CA) then suspended in Bind and Wash Buffer (BWB) (5 mM Tris-HCl, 0.5 mM EDTA, 1.0 M NaCl, pH 7.5). Beads were then incubated in BWB for 30 min at room temperature in the presence of 10 uM oligonucleotides (Table 1). These oligonucleotides were modified with a dual biotin group at the 5' end with the biotin groups separated by a six-carbon linker (IDT, Coralville, IA). After binding, the beads were washed 3 times with 1x PCR buffer to thoroughly remove unbound oligonucleotides.

Step 2 – Preparing microemulsions. Microemulsions for PCR were prepared by slight modifications of previously described methods (14) (15). The oil phase was composed of 4.5% Span 80 (S6760, Sigma, St. Louis, MO), 0.40 % Tween 80 (Sigma S-8074), and 0.05% Triton X-100 (Sigma T-9284) in mineral oil (Sigma M-3516). The oil phase was freshly prepared each day. The aqueous phase consisted of 67 mM Tris-HCl (pH 8.8), 16.6 mM NH4SO4, 6.7 mM MgCl2, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 0.05 uM forward primer, 25 uM reverse prime, 45 umits Platinum Taq (Invitrogen 10966-034), various amounts of template DNA (see results), and ~10^8 oligonucleotide-coupled beads in a total volume of 300 ul. The "forward" primer was an oligonucleotide whose sequence was identical to the 3' 20 – 22 nt of that described in step 1 and was not modified with biotin.

Water-in-oil microemulsions were prepared by drop wise addition of 200 uL of the aqueous phase to 400 ul of the oil phase previously placed in a 2 ml round bottom cryogenic vial (product # 430661, Corning, Corning, NY). The drop wise addition was performed over ~ one

minute while the mixture was being stirred at 1400 RPM with a magnetic microstir bar (product # 58948-353, VWR, address) on a VWR model 565 Magnetic Stirrer. After the addition of the aqueous phase, the mixture continued to be stirred for a total time of 30 minutes. Two emulsions were made at once by placing two tubes in a rack placed at the center of the magnetic stirrer.

Step 3 - PCR cycling. The emulsions were aliquotted into five wells of a 96 well PCR plate, each containing 100 ul. PCR was carried out under the following cycling conditions: 94°C for 2 minutes; 40 cycles of 94°C for 15 seconds, 57°C for 30 seconds, 70°C for 30 seconds. The PCR products analyzed in this study ranged from 189 to 239 bp.

Step 4 – Magnetic capture of beads. After PCR cycling, the microemulsion from five wells of a PCR plate were pooled and broken by addition 800 ul of NX buffer (100 mM NaCl containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) in a 1.5 ml tube (Corning, product # 430909). After vortexing for ~20 sec. the beads were pelleted by centrifugation in a microcentrifuge at 8000 rpm (5000 g) for 90 seconds. The top oil phase and all but ~300 ul of the aqueous phase was removed from the tube and 600 ul of NX buffer was added. After vortexing for 20 sec. and centrifugation for 90 sec., the top oil phase and all but ~300 ul of the aqueous phase was removed. The addition of 600 ul NX buffer, vortexing, and centrifugation was repeated once more and the top oil portion and all but ~300 ul of the aqueous phase was removed. The tube was then placed on a magnet (Dynal MPC-S)and the rest of the supernatant was carefully pipetted off. The beads were washed an additional 3 times with 1x PCR buffer using magnetic separation rather than centrifugation and finally resuspended in 100 ul of 1x PCR buffer.

Step 5 – Sequence differentiation. Two oligonucleotide probes were used for each reaction. One was 5'-labeled with 6-carboxyfluorescein (6-FAM) and was specific for one allele and the second was 5'-labeled with biotin and was specific for the other allele. Probes were synthesized by IDT. The 30 ul hybridization reactions contained 10 uM of each probe and 5 - 25 million beads in1 x PCR buffer. Reactions were performed in PCR plates on a thermal cycler by heating to 94°C for 30 seconds then cooling to 75°C at a rate of 0.5°C per second, cooling to 45°C at 0.2°C per second, and finally cooled to 30°C at 1°C per second. All subsequent steps were performed at room temperature. The reactions were transferred to a 96 well Costar plate (Corning product # 3797) and placed on a 96 well magnet. Beads were collected magnetically by exposing them to the magnet for 2 minutes. The supernatant was removed and the beads washed 3 times with 1x PCR buffer by pipetting them and collecting for two minutes. They were finally resuspended in 100 ul B-PCR buffer (1mg/mL BSA in 1x PCR buffer). The beads were then incubated for 10 minutes in a total volume of 100 ul B-PCR buffer containing 3 ug of Alexa-488 rabbit anti-fluorescein antibody (Molecular Probes product # A-11090, Eugene, OR) and 3 ug of Nutravidin labeled with phycoerythrin (Molecular Probes product # A-2660) in B-PCR buffer The beads were washed three times and resuspended in B-PCR buffer as described above. They were then incubated for ten minutes in a total volume of 100 ul B-PCR buffer containing 6 ug of Alexa 488- conjugated chicken anti-rabbit antibody (Molecular Probes product # A-21441)and 3 ug of biotinylated goat anti-avidin antibody (Vector Laboratories, product # BA-0300, Burlingame, CA). The beads were washed three times and resuspended in B-PCR buffer as described above. They were then incubated for ten minutes in a total volume of 100 ul B-PCR buffer containing 3 ug of an Alexa 488-conjugated goat anti-chicken antibody (Molecular Probes product # A-11039) and 3 ug of phycoerythrin-labeled streptavidin (Molecular Probes product # S-866). This solution was then washed an additional 3 times with 1x PCR buffer and resuspended in 20 ul of 1 X PCR buffer.

Step 6 – Flow Cytometry. The bead suspension was diluted to a concentration of ~ 10^6 - 10^7 beads per ml in 10 mM Tris-HCl, 1 mM EDTA (product #351-010-131, Quality Biological, Inc., Gaithersburg, MD) and analyzed using a LSR instrument (BD Biosciences, Franklin Lakes, NJ). The instrument was set up for standard two-color analysis using an argon laser and optical filters that distinguished between the two fluorescent dyes. No spectral deconvolution was required as the major bead populations were well-separated. In some cases, scanning was performed with FACScan or FACS Calibur instruments (BD Biosciences), yielding equivalent results. Sorting was carried out with a FACS Vantage SE instrument (BD Biosciences).

Template preparation and sequence analyses. Human genomic DNA was purified with DNeasy (product #69504, Qiagen, Valencia, CA). RNA was purified with Quickprep (product # 27-9255-01, Amersham Biosciences, Piscataway, NJ). Reverse transcription of RNA was performed using Superscript II reverse transcriptase (Invitrogen #18064014) according to the manufacturer's instructions. PCR using genomic DNA or reverse transcripts as templates was performed as described (7). PCR products to be used as templates for BEAMing or for sequencing were purified with QIAquick (Qiagen product # 28104). Sequencing reactions were performed using Big Dye v3.0 reagents (Applied Biosystems, Foster City, CA) and analyzed by capillary electrophoresis (Spectrumedix 9600, State College, PA).

Results

BEAMing consists of the six steps diagrammed in Fig. 1.

Step 1 - Coupling oligonucleotides to beads. - We used streptavidin-beads because of the simplicity of coupling biotinylated oligonucleotides to them. Oligonucleotides with just a single 5' biotin group were found to dissociate from the beads during temperature cycling, while oligonucleotides labeled with dual biotin groups at their 5' end (separated by a six-carbon linker) were stable to cycling. As determined by fluoroscopic measurements of oligonucleotides doubly labeled with 6-FAM and biotin, ~10^5 oligonucleotide molecules were bound to each bead. We found that short oligonucleotides (20 bases) did not work as well for priming as longer ones (41 bp), perhaps because of steric hindrance at the bead surface. It is likely that amino-, sulfhydryl-, or carboxyl-modified oligonucleotides covalently coupled to beads modified with corresponding reactive groups could also function as bead-bound primers for BEAMing.

Step 2 – Preparing microemulsions. The size of the individual aqueous compartments ranged from less than 1 micron to >10 microns in diameter (Fig. 2). We estimated that an emulsion comprising 200 ul of aqueous solution and 400 ul of oil would contain ~3 x 10^9 compartments of average diameter 5 microns. Approximately 10^8 beads were included in each emulsion, so that only one in ~30 compartments contained a bead. The optimal amount of template was experimentally determined to be ~ 5 x 10^8 molecules, so that one in ~six compartments contained a template molecule.

Step 3 - PCR cycling. PCR priming by oligonucleotides coupled to beads was found to be very inefficient compared to the priming by the same oligonucleotides when free in solution. For this reason, a small amount of non-biotinylated forward primer identical in sequence to the biotinylated oligonucleotide coupled to the beads was included in the reactions, This facilitated the first few rounds of amplification of the single template within each aqueous compartment. In the absence of additional primer, no detectable amplification on the beads was generated. Conversely, if too much additional primer was included, no amplification on the beads occurred because of competition with the primers in solution. An excess of the

reverse primer was included in the aqueous compartment to maximize the probability that bead-bound oligonucleotides extended by polymerase would serve as templates for further amplification cycles.

Step 4 – Magnetic capture of beads. There are several ways to break water-in-oil emulsions, including extraction with organics (14). We found that simply adding non-ionic detergents produced phase separations without any detectable modification of the beads or DNA molecules bound to them.

Step 5 – Sequence differentiation. Most fluorescence-based methods for distinguishing alleles in homogeneous or two-phase assays can be used to assess allelic variation captured on beads. These methods include single nucleotide extension, allele specific priming, or hybridization. We generally employed hybridization of fluorescein-conjugated or biotin-conjugated oligonucleotides for discrimination. As shown in Fig. 1 and Table 1, these oligonucleotides had a stem-loop structure, with the middle of the loop containing the variant nucleotide(s) (see Table 1). This design was based on studies of Molecular Beacons wherein a stem-loop structure was shown to markedly improve allelic discrimination (16). The oligonucleotides we used differed from Molecular Beacons in that there was no need for a quenching group. Such quenching is required for homogeneous assays when unhybridized oligonucleotides cannot be removed from the reactions prior to assay but is not necessary for solid phase assays such as those employed with beads.

Step 6 – Flow Cytometry. Optimum results in flow cytometry depend on high fluorescent signals on the beads. We generally enhanced the fluorescence emanating from the hybridization probes with secondary reagents. For example, Alexa 488—labeled antibodies were used to enhance the signals emanating from fluorescein-coupled oligonucleotide probes. Similarly, phycoerythrin-labeled streptavidin was used to generate a signal from biotin-labeled oligonucleotide probes. Flow cytometers equipped with two or three lasers and appropriate filters have the capacity to distinguish multi-allelic loci and to perform multiplex analysis of several genes simultaneously. The newest generation of flow cytometers can also analyze >50,000 particles per second. In addition to the analytical power of flow cytometry, FACS instruments can separate specific populations of beads for further analysis.

Characteristics of microemulsions. Pilot experiments demonstrated that simply stirring the water-oil mixtures described in Materials and Methods produced very stable microemulsions of a size compatible with that of the beads. In the experiment shown in Fig. 2, the aqueous compartment contained a blue dye and 1 micron magnetic beads that were labeled by binding to an oligonucleotide that was biotinylated at its 5' end and labeled with fluorescein at its' 3' end. The appearance of emulsions immediately after their formation is shown in Fig. 2. As expected, this appearance was unchanged after temperature cycling during PCR (15). Most aqueous compartments contained no beads, as expected from the figures provided in the previous section. Those compartments that did contain beads generally contained only one, though a fraction contained more, as expected from a Poisson distribution and non-uniform aqueous compartment sizes. "Heterozygous" beads containing PCR products representing both alleles are produced when two or more DNA template molecules are contained within a single aqueous compartment. Such heterozygotes can compromise the accuracy of the analyses under some circumstances (see Discussion).

Detection of homozygotes and heterozygotes. Fig. 3 shows typical results obtained with human DNA samples. The MID42 marker used in this experiment was chosen from a collection of diallelic short insertion/deletion polymorphisms assembled by Weber and

colleagues (17). These alleles are particularly simple to distinguish with hybridization probes because the two alleles at each locus differ by ~ 4 bases. The probe for the longer (L) allele was labeled with fluorescein (green) and the probe for the shorter (S) allele labeled with phycoerythrin (red).

Fig. 3*A* shows a plot of the side scatter vs. forward scatter of beads following BEAMing. In general, >75% of beads were dispersed as single particles, with the remainder aggregated in groups of two or more. Subsequent flow cytometric analysis was confined to the singlet beads, gated as outlined in Fig. 3*A*.

Figs. 3*B* - *D* show density plots of gated beads generated with various templates. In Fig. 3*B*, a template from an individual homozygous for the L allele was included in the emulsion. Two populations of beads were apparent. 98 % of the beads contained no PCR product (black) and the remaining 2% fluoresced in the FL1 channel (colored green in Fig. 3). Fig. 3*C* represents the analysis of an individual homozygous for the S allele. Two populations of beads were again apparent, but this time the labeled population fluoresced in the FL2 channel (colored red in Fig. 3). Fig. 3*D* presents density plots from the analysis of an individual heterozygous at the MID42 locus. Four populations of beads are evident: the black region represents beads without any PCR product, the red region represents beads containing PCR products from the L allele, the green region represents beads containing PCR products from the S allele, and the blue region represents beads containing PCR products from both alleles. Beads containing PCR products from both alleles were derived from aqueous compartments which contained more than one template molecule. The number of blue beads increased in a non-linear fashion as more template molecules were added. At the extreme, when all aqueous compartments are saturated, virtually all beads will register as blue. Operationally, we found that the bead populations were most distinct when the number of beads containing any PCR product was < 10% of the total beads analyzed.

PCR products vs. genomic DNA or cDNA as templates. The results shown in Fig. 3 were generated using PCR products made from human genomic DNA samples. As the ratio of the beads represent L alleles to those representing S alleles was 1.0 in this experiment, it was clear that the initial PCR did not preferentially amplify either allele. The use of PCR products rather than genomic DNA permitted large numbers of alleles to be amplified from even small quantities of starting DNA. In general, 10 to 100 pg of PCR products of size 200 bp were found to be optimal for BEAMing, producing PCR-mediated extension of primers on ~1 to 10% labeled beads.

In some situations it might be useful to use genomic DNA rather than PCR products as templates for BEAMing. The data in Fig. 4*A* and *B* show flow cytometric data from an experiment wherein 10 ug or 1 ug of human genomic DNA was used as template for BEAMing at the MID42 locus. Patterns very similar to those shown in Fig. 3 were observed, though fewer beads were labeled than when PCR products were used as templates.

BEAMing could also be used to analyze variations in expression from the two alleles of a heterozygous individual. Heritable variations in the expression from individual alleles of the same gene have been shown to occur often in humans (18) and mice (19) and can have significant phenotypic effects (20). The results shown in Fig. 4*C* and *D* show that PCR products made from reverse-transcribed mRNA can be used for BEAMing. In this case, calpain-10 transcripts differing by a single nucleotide polymorphism (SNP) were analyzed. For SNPs like these, probes that incorporated an extra mismatched nucleotide adjacent to the polymorphic nucleotide (see Table 1) can enhance the distinction between alleles (21)

(22). The results from two independent emulsions made with aliquots of the same RT-PCR product are shown to illustrate reproducibility. Though the number of beads that functioned as templates in BEAMing varied up to 3-fold among experiments with identical templates, the proportion of beads representing the two alleles was reproducible (775 A allele beads to 690 G allele beads in Fig. 4*C* and 1380 A allele beads to 1227 G allele beads in Fig. 4*D*) respectively).

Analysis of minor variants in a DNA population. The analysis of uncommon variations is ideally suited for analysis via BEAMing because of the large number of molecules that can be independently analyzed while retaining a high signal-to-noise ratio. Fig. 5*A* shows representative data from templates representing 1%, 2%, 3%, and 4% of the L allele of MID42. The linearity and precision of these measurements, with correlation coefficient of 0.99, demonstrates the utility of this approach for such applications. We also applied this analysis to the detection of *KRAS* and could easily observe 0.1% mutants when spiked into a population of wt molecules (data not shown).
The rare beads representing the mutant alleles could not only be quantified but could also be purified for subsequent analysis. As demonstration, samples of the beads enumerated in Fig. 5*A* were additionally assessed using a flow cytometer equipped with sorting capabilities. Beads were sorted and individual beads used as templates for conventional PCR using the same primers employed for BEAMing. As each bead contains thousands of bound template molecules, single beads were expected to generate robust PCR products (23). and this was experimentally confirmed. These PCR products were then subjected to sequencing. As shown in Fig. 5*B* and *C*, green and red beads generated PCR products exclusively of the L and S types, respectively.

Discussion

The results described above show that BEAMing provides a reliable and sensitive assay for measuring variations in genes and transcripts. It requires no instrumentation other than a magnetic stirrer, a temperature cycler and a flow cytometer, all of which are widely available. There are several other advantages of this approach. First, the sensitivity can be increased to meet the specifications of the experiment simply by analyzing more beads. Such sensitivity is limited only by the error rate of the polymerases used for amplification. Second, BEAMing data can be used not only to demonstrate that a variant is present in a particular population of DNA molecules, but also quantifies the fraction of variant DNA molecules in that population (Fig. 5*A*). Such quantification is not possible with techniques that destroy or ignore the wild type molecules as part of the assay, such as those that use allele specific priming or endonuclease digestion during PCR. Third, the beads containing variant alleles can easily purified through flow sorting and use for subsequent experimentation. Such recovery is difficult with digital techniques that count molecules deposited on microscope slides. And finally, the BEAMing approach is in principle automatable.

Several modifications of the basic principles described here can be envisioned that will further simplify the technology or widen its applications. For example, microemulsions were made by stirring water/oil/detergent mixes. The sizes of the resultant aqueous compartments were somewhat heterogeneous, as illustrated in Fig. 2. A relatively large number of beads containing PCR products of both alleles are obtained from large compartments because they are more likely to contain >1 template molecule than smaller compartments. Though this is not a problem for the analysis of uncommon variants, it does pose a problem when the

variant to be analyzed is present in a substantial fraction of the DNA molecules. For example, it is easy to distinguish a population containing 2% of allele A and 98% of allele B from one that contains 0% of allele A (Fig. 5*A*). But it is more difficult to distinguish a population that contains 48% of allele A and 52% of allele B from a population that contains 50% of allele A; the large number of "heterozygote" beads formed in the latter analysis diffuse the boundaries of the pure red and green channels. This limit to accuracy can theoretically be overcome through the preparation of more uniformly sized aqueous compartments, perhaps through sonication or pressure-driven emulsifiers.

Though flow cytometry requires only seconds to minutes per sample, multiple parallel analyses could facilitate throughput. Novel particle counting designs may prove useful for this purpose. Another way to increase throughput would be to physically separate the beads that contained PCR products prior to flow cytometry. This could be accomplished with antibodies to modified nucleotides incorporated into the PCR product during BEAMing.

Though we have focused on issues related to human variation, BEAMing technology could obviously be applied to genes or transcripts of any organism or population of organisms. It could also be used to quantify epigenetic alterations, such as methylation, if DNA was first treated with bisulfite to convert methylated cytosine residues to thymidine. Beads generated from random fragments of whole genomes (24), rather than from individual genes as described above, could be used to identify gene segments that bind to specific DNA-binding proteins (25). And if the beads made by BEAMing were used in compartmentalized in vitro transcription-translation reactions, variant proteins would be bound to beads containing the corresponding variant DNA sequences (23). This could allow facile flow cytometric evaluation of rare mutations using antibodies that distinguished between wt and mutant gene products (26).

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Figure Legends

FIG. 1 Schematic of BEAMing. Step 1- Magnetic beads covalently coated with streptavidin are bound to a biotinylated oligonucldotide ("oligos"). Step 2 - An aqueous mix containing all the necessary components for PCR plus primer-bound beads and template DNA are stirred together with an oil/detergent mix to create microemulsions. The aqueous compartments (white circles in the gray oil layer) contain an average of <1 template molecule and <1 bead. Red and green templates represent two template molecules whose sequences differ by one or many nucleotides. Step 3 - The microemulsions are temperature cycled as in a conventional PCR. If a DNA template and a bead are present together in a single aqueous compartment, the bead bound oligonucleotides act as primers for amplification. The straight red and green lines connected to the beads represent extension products from the two different kinds of templates. Step 4 - The emulsions are broken and the beads are purified with a magnet. Step 5 - After denaturation, the beads are incubated with oligonucleotides that can distinguish between the sequences of the different kinds of templates. Fluorescently-labeled antibodies are then used to label the bound hybridization probes. This renders the beads containing PCR product as red or green upon appropriate laser excitation. Step 6 - Flow cytometry is used to count the red and green beads.

FIG. 2 Photograph of a typical microemulsion. Microemulsions were made as described in Materials and Methods with the exception that the aqueous compartments contained cascade blue-labeled dCTP and the beads were pre-labeled with phycoerythrin (red) or Alexa 488 (green). One ul of microemulsion was deposited in 1 ul of oil on a microscope slide prior to photography. Of the seven aqueous compartments visible in this picture, two contain beads. Note the heterogeneous size of the aqueous compartments (beads are 1.05 microns in diameter).

FIG. 3 Density plots of flow cytometric data obtained from BEAMing. The locus queried in this experiment was MID42 and PCR products generated from genomic DNA were used as templates in the microemulsions. (A) Forward scatter (FSC) and side scatter (SSC) of all beads show that ~80% of the total beads are singlets, with most of the remaining beads aggregated as doublets, The "noise" is instrumental and is observed with blank samples containing no beads. The instrument output was gated so that only singlets were analyzed for fluorescence analysis. The patterns observed from an individual homozygous for the L allele (A), homozygous for the S allele (B), and heterozygous for L and S (D) are shown in (B), (C), and (D), respectively. The regions containing beads hybridizing to the L and S allele probes are labeled green and red, respectively. The region containing beads that did not hybridize to any probe is black and the region containing beads that hybridized to both probes is blue. The blue beads arose from aqueous compartments in which both types of template molecules were present. The proportion of singlet beads that hybridized to at least one of the probes was 2.9%, 4.3%, and 20.3% in (B) to (D), respectively.
The FSC and SSC plots in (A) represent the same beads analyzed in (D).

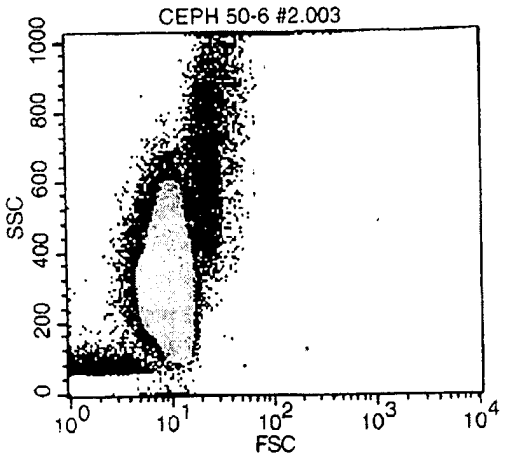
FIG. 4 Density plots of BEAMing using genomic DNA or RT-PCR products as templates. The data in (A) and (B) were generated by including 10 and 1 ug of human genomic DNA, respectively, in the microemulsions, querying the *MID42* locus. The data in (C) and (D) were generated using emulsions that contained ~ 50 pg of PCR products synthesized from cDNA of lymphoblastoid cells, querying the *calpain-10* locus. The green and red regions correspond to the L and S alleles for *MID42* and to the A and G alleles for *calpain-10.* The number of beads in the outlined regions containing red and green beads are shown in each case. The proportion of singlet beads that hybridized to at least one of the probes was 1.2%, 0.6%, 6.8% and 4.2% in (A) to (D), respectively. The outlined regions used for counting in (A) and (B) were identical, as were those used for (C) and (D). Beads that did not hybridize to any probe were gated out and therefore not evident in the graphs, while the region containing beads that hybridized to both probes is labeled blue.

Fig. 5 Detection and validation of variants present in a minor fraction of the DNA population. (A) Mixtures of PCR products containing 0% to 4% L alleles of MID42 were used for BEAMing. Flow cytometry such as that shown in Fig. 3 was used to determine the fraction of singlet beads that were red (y-axis). The proportion of singlet beads that hybridized to at least one of the probes varied from 3.2% to 4.3%. (B) Beads were sorted with the FACS Vantage SE instrument and individual red or green beads were used as templates for conventional PCR employing the forward and reverse primers listed in Table 1. Red beads generated only the S allele sequence while green beads generated only the L allele sequence.

Table 1 Oligonucleotides

Locus	Oligonucleotide*	Modification	Use**
MID42	5'-tactatgtatttatacgttaagacctctatgaatgaatgta	5' Dual biotin	Bound to Beads
MID42	5'-cgttaagacctctatgaatgaatgta	none	Forward Primer for PCR
MID42	5'-gaaaggtaagtacagggaaagg	none	Reverse Primer for PCR
MID42	5'-cacgcagattgaattaaacAGTTagttacaaagacacgtg	5' 6-FAM	Hybridization probe for L allele
MID42	5'-cacgcagattgaattaaacagttacaaagacacgtg	5' Biotin	Hybridization probe for S allele
Calpain-10	5'-aggtcccagagggtggaaggagccaggacgcacccccactgctgctg	5' Dual Biotin	Bound to Beads
Calpain-10	5'-aggtcccagagggtggaag	none	Forward Primer for PCR
Calpain-10	5'-ttgcgatggtcactgtgaag	none	Reverse Primer for PCR
Calpain-10	5'-cacggtaggtgctTgcaggcagcgtg	5' 6-FAM	Hybridization probe for A allele
Calpain-10	5' -cacggtaggtgccCgcaggcagcgtg	5' Biotin	Hybridization probe for G allele
KRAS2	5'-ttcgtccacaaaatgattctgaattagctgtatcgtcaagg	5' Dual Biotin	Bound to Beads
KRAS2	5'-agaatggtcctgcaccagtaa	none	Reverse Primer for PCR
KRAS2	5'-catgttctaatatagtcacattttca	none	Forward Primer for PCR
KRAS2	5'-cacgggagctGGTGGCgtagcgtg	5' 6-FAM	Hybridization probe for wt allele
KRAS2	5'-ccacgggagctgatggcgtagcgtgg	5' Biotin	Hybridization probe for mutant allele

*Bases in upper case represent allelic differences.
**Hybridization probes each contained 4 bases at their 5' and 3' ends to form hairpins, as explained in the text.



File: CEPH 50-6 #2.003
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Total Events: 110521

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Green Gate	777	0.80
Red Gate	775	0.80
R4	4126	4.23

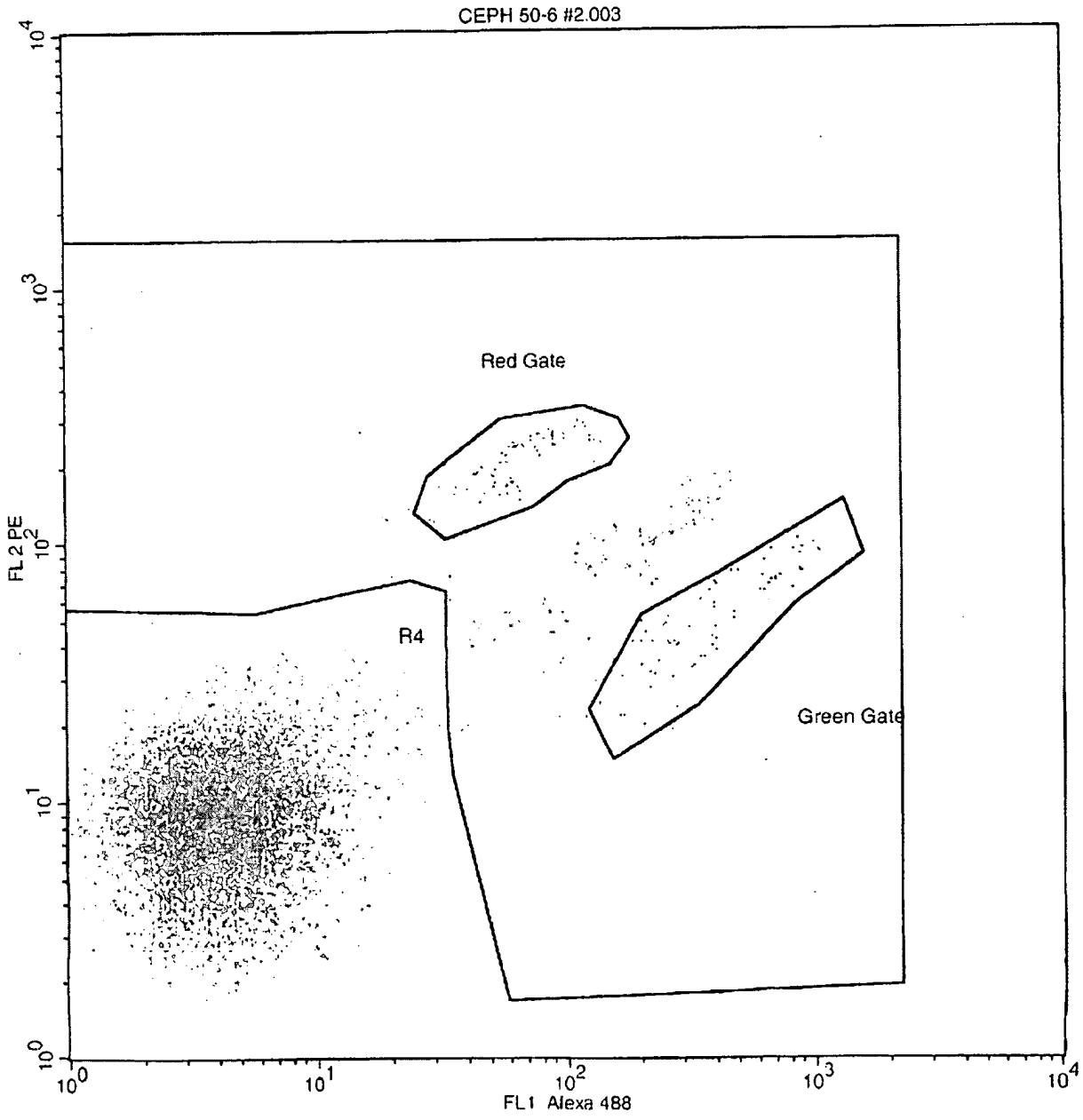


Exhibit E

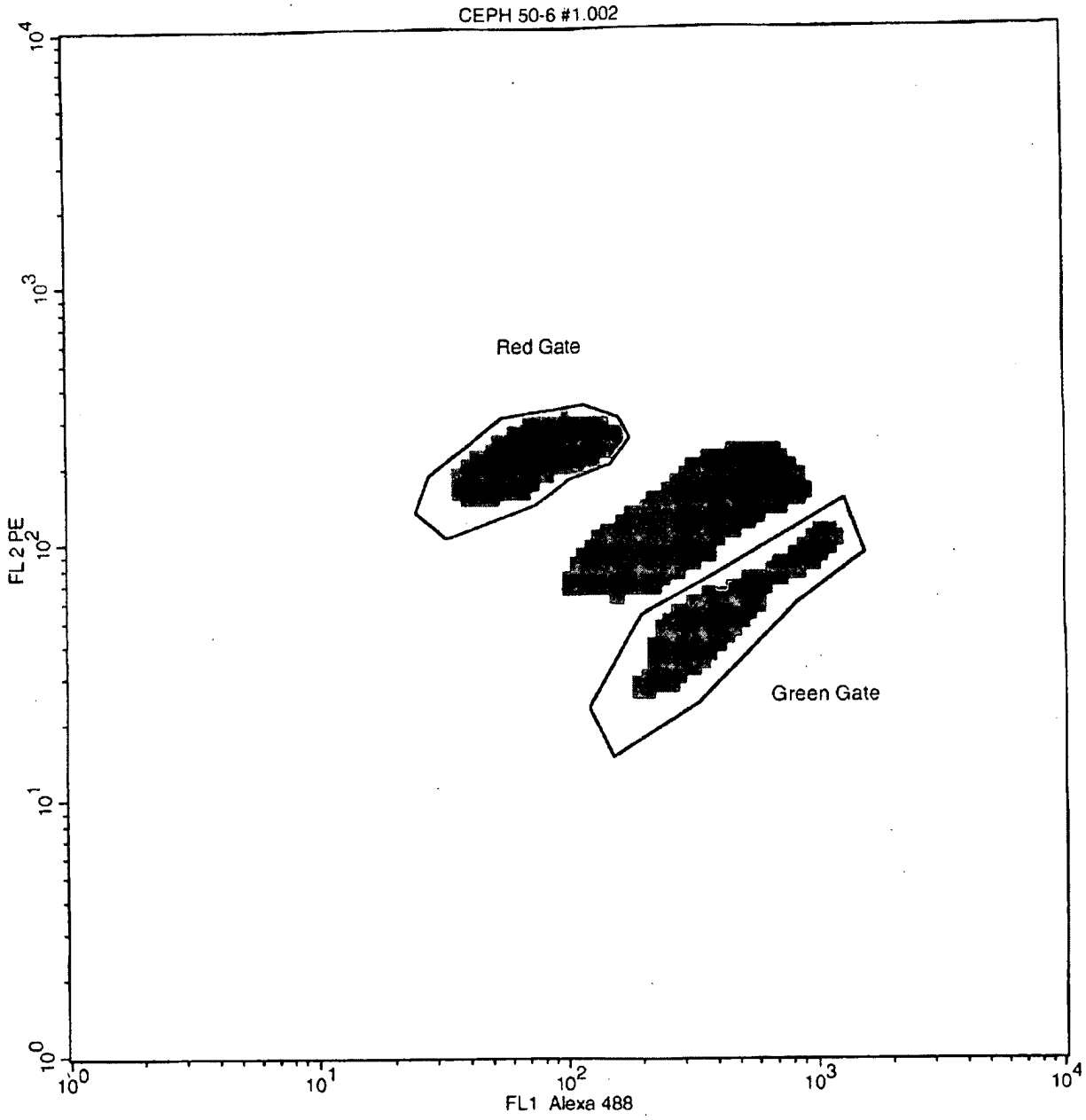
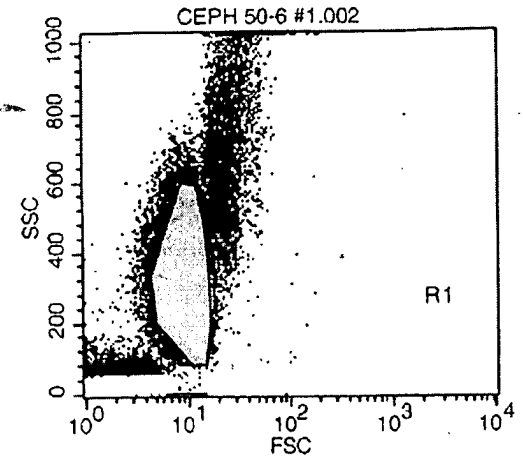

CEPH 50-6 #1.002
Red Gate
Green Gate
FL2 PE
FL1 Alexa 488
10^0
10^1
10^2
10^3
10^4

Exhibit F



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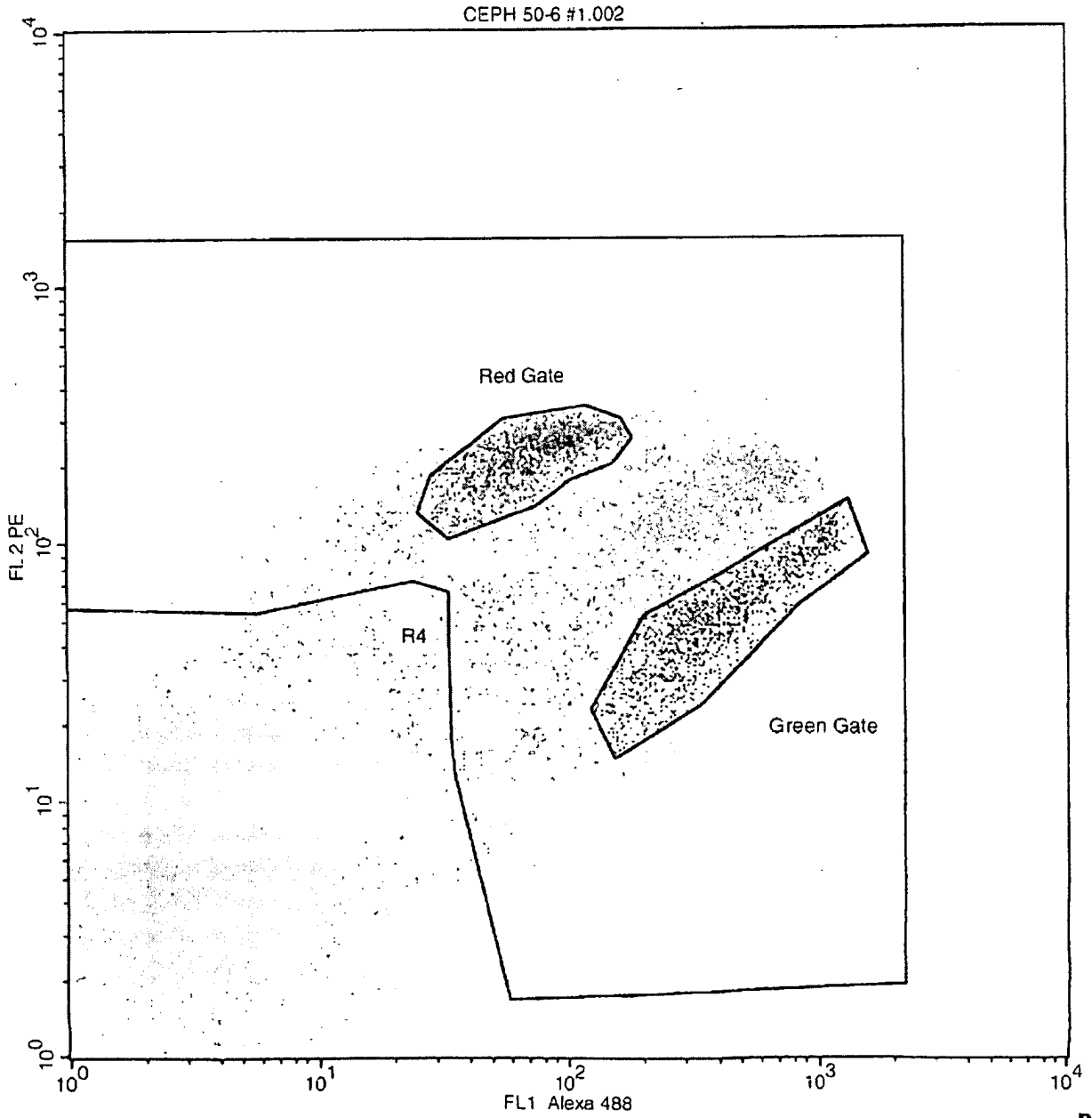
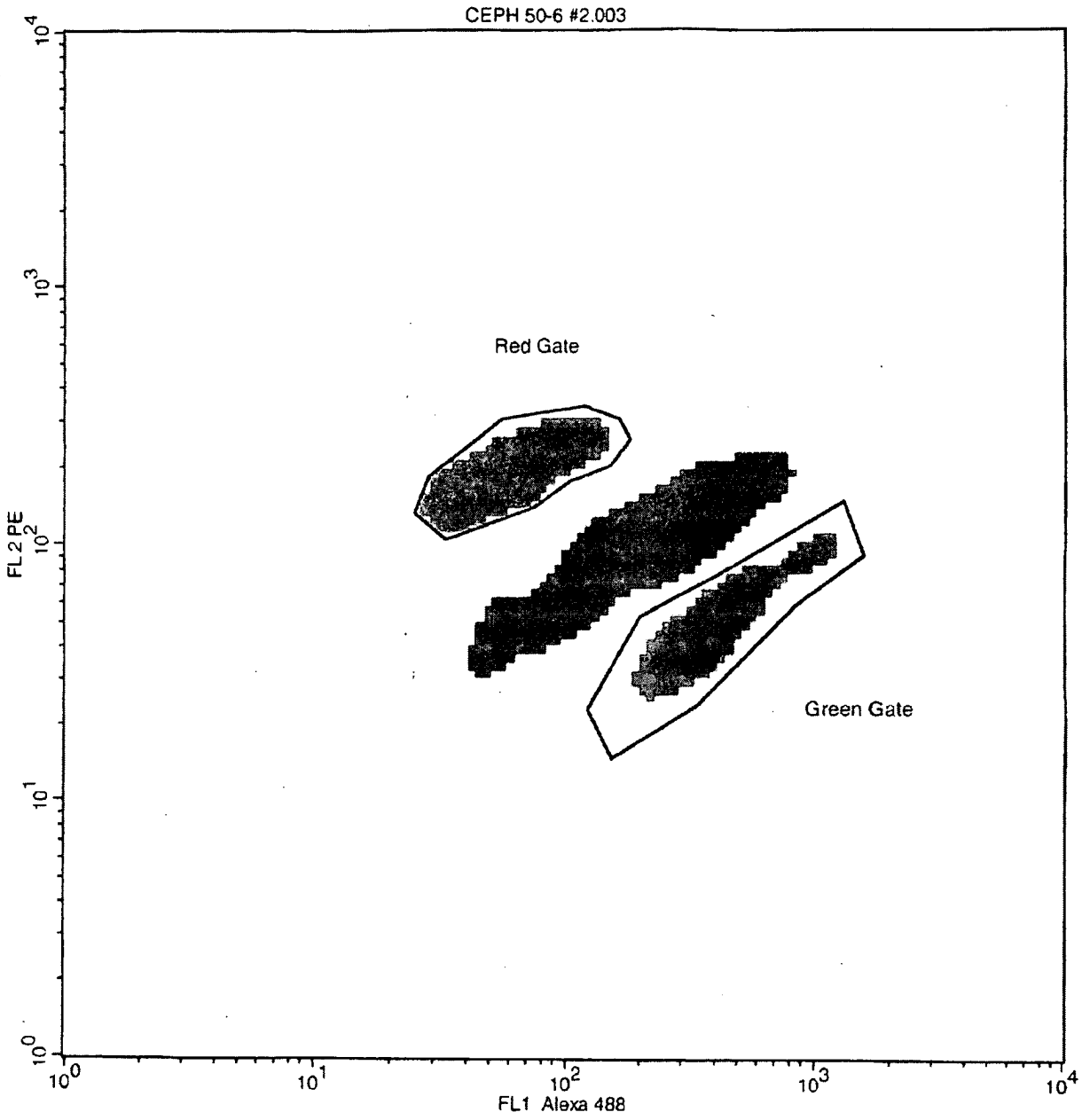
R1	97786	100.00
Green Gate	1383	1.41
Red Gate	1380	1.41
R4	6651	6.80

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Green Gate
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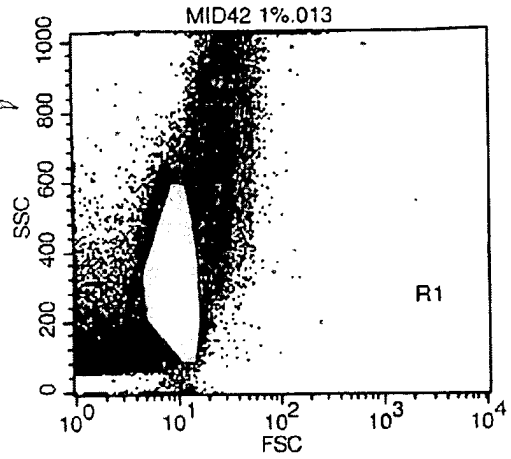
FL1 Alexa 488
10^0
10^1
10^2
10^3
10^4

Exhibit H



File: MID42 1%.013
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Region	Events	% Gated
R1	195959	100.00
Green Gate	7288	3.72
Red Gate	73	0.04

0.99 %

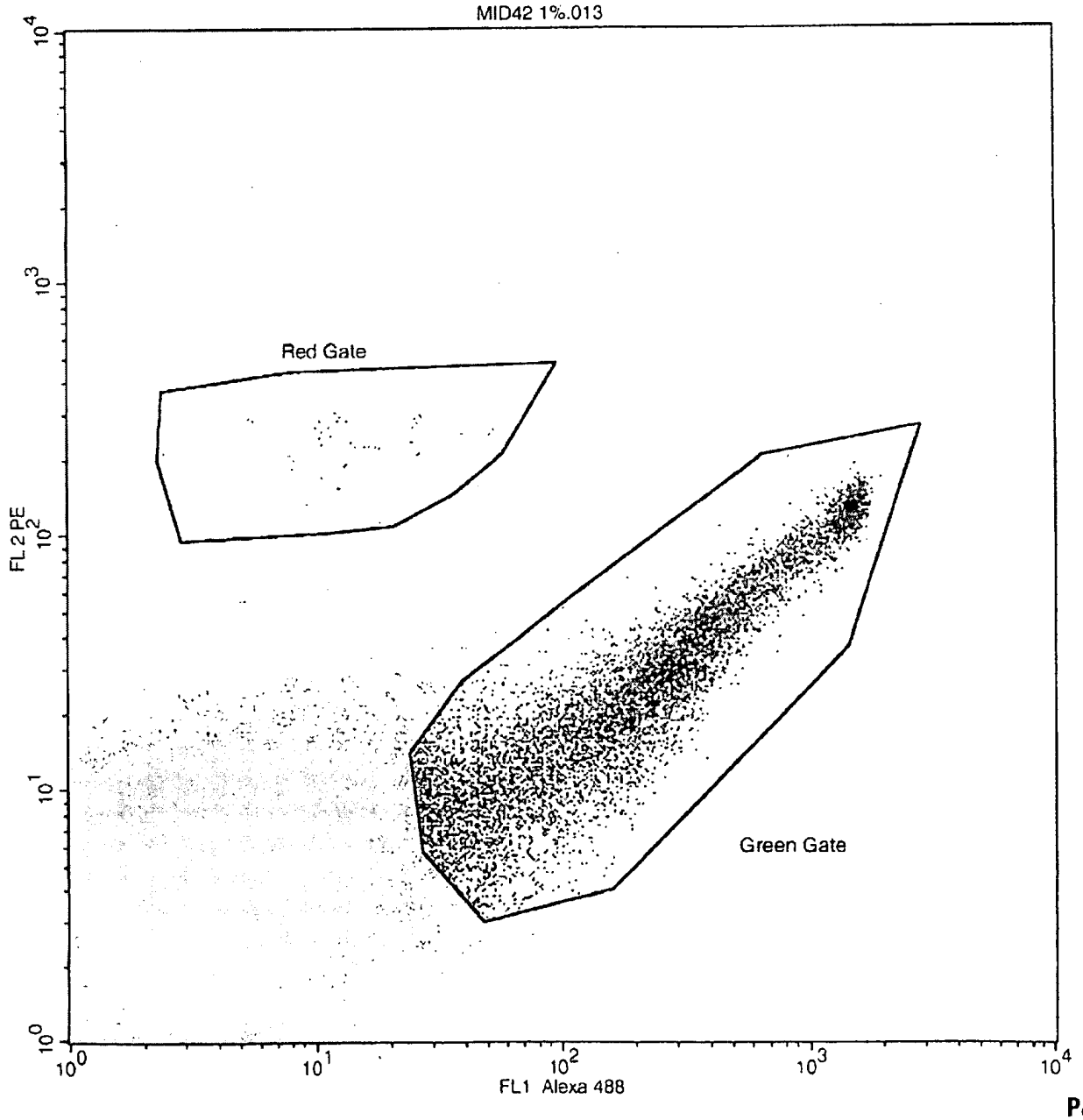
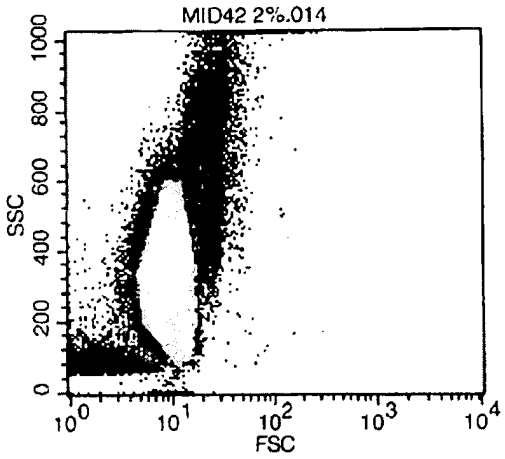


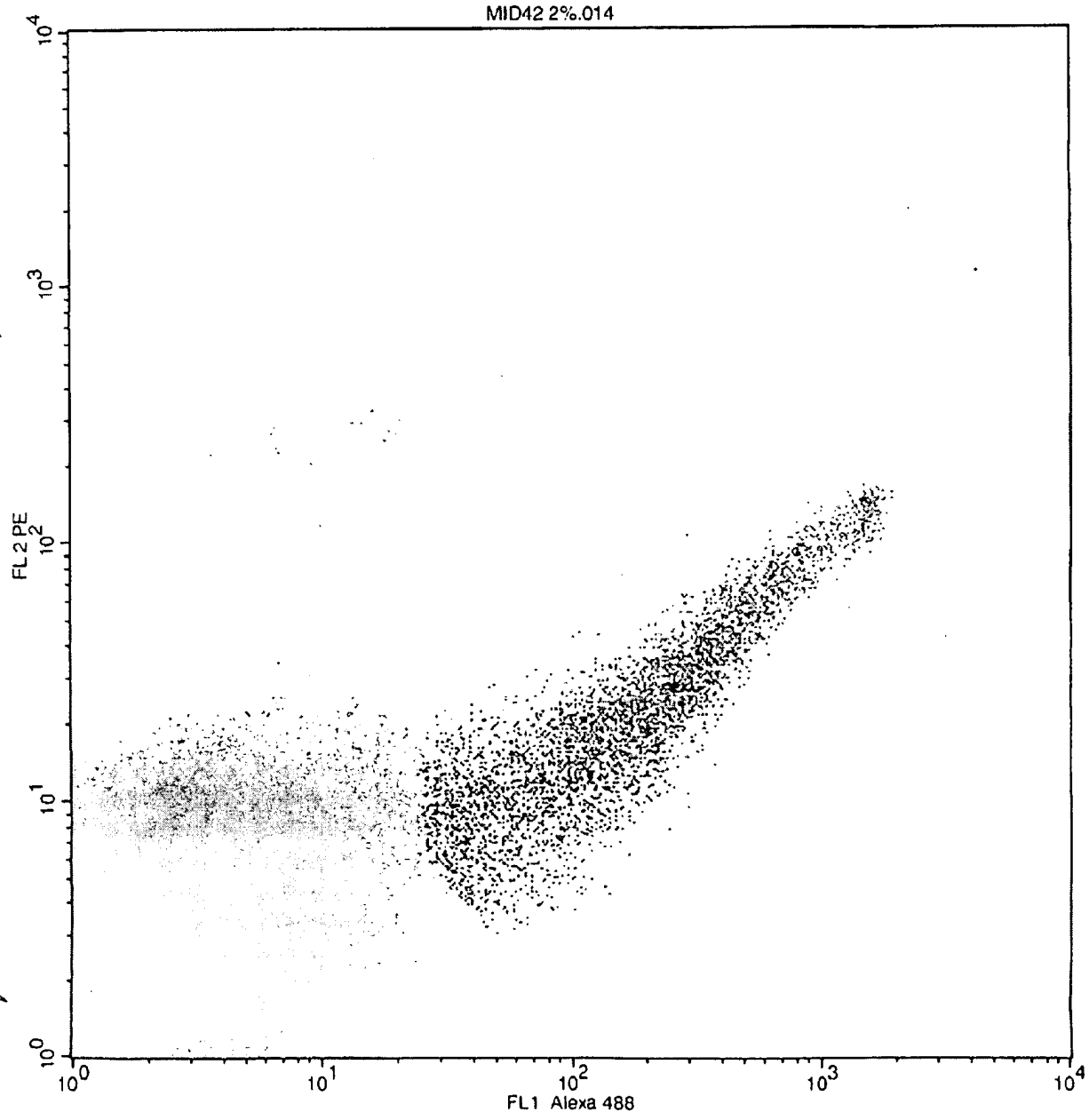
Exhibit I



File: MID42 2%.014
Acquisition Date:
Total Events: 178887

Region	Events	% Gated
R1	151384	100.00
Green Gate	4525	2.99
Red Gate	90	0.06

1.9 [illegible]%



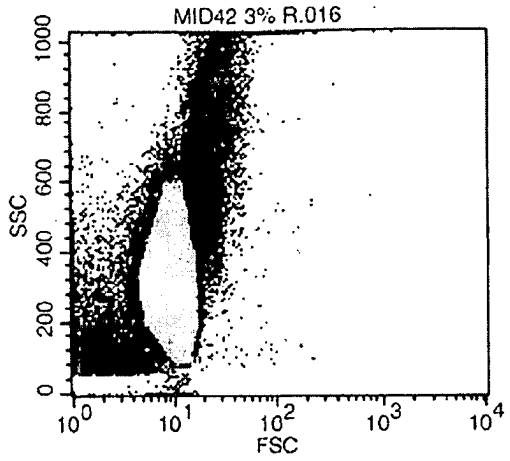


Exhibit J

File: MID42 3% R.016
Acquisition Date:
Total Events: 189867

Region	Events	% Gated
R1	154781	100.00
Green Gate	5717	3.69
Red Gate	192	0.12

3.2%

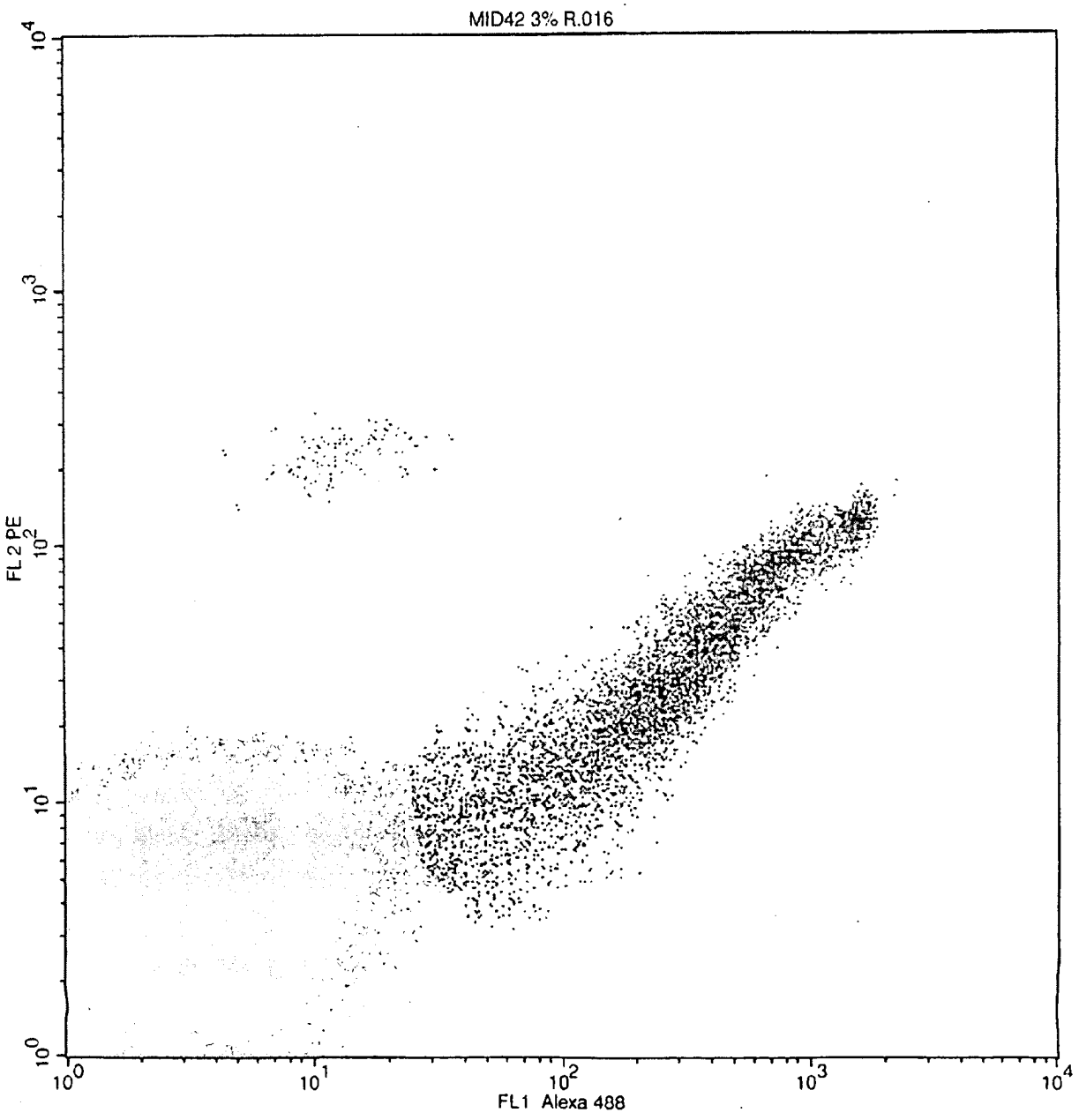
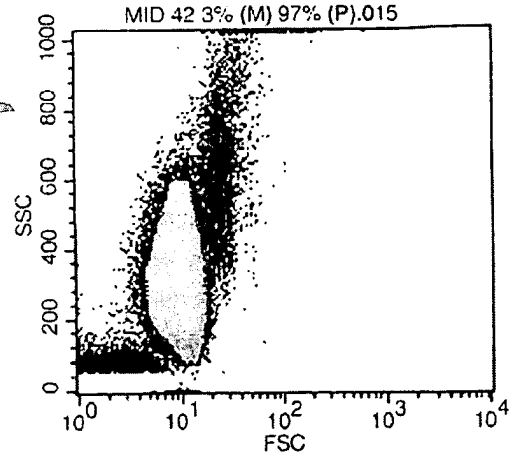


Exhibit K



File: MID42 4% .017
Acquisition Date:
Total Events: 147377

Region	Events	% Gated
R1	121113	100.00
Green Gate	4802	3.96
Red Gate	202	0.17

4.03%

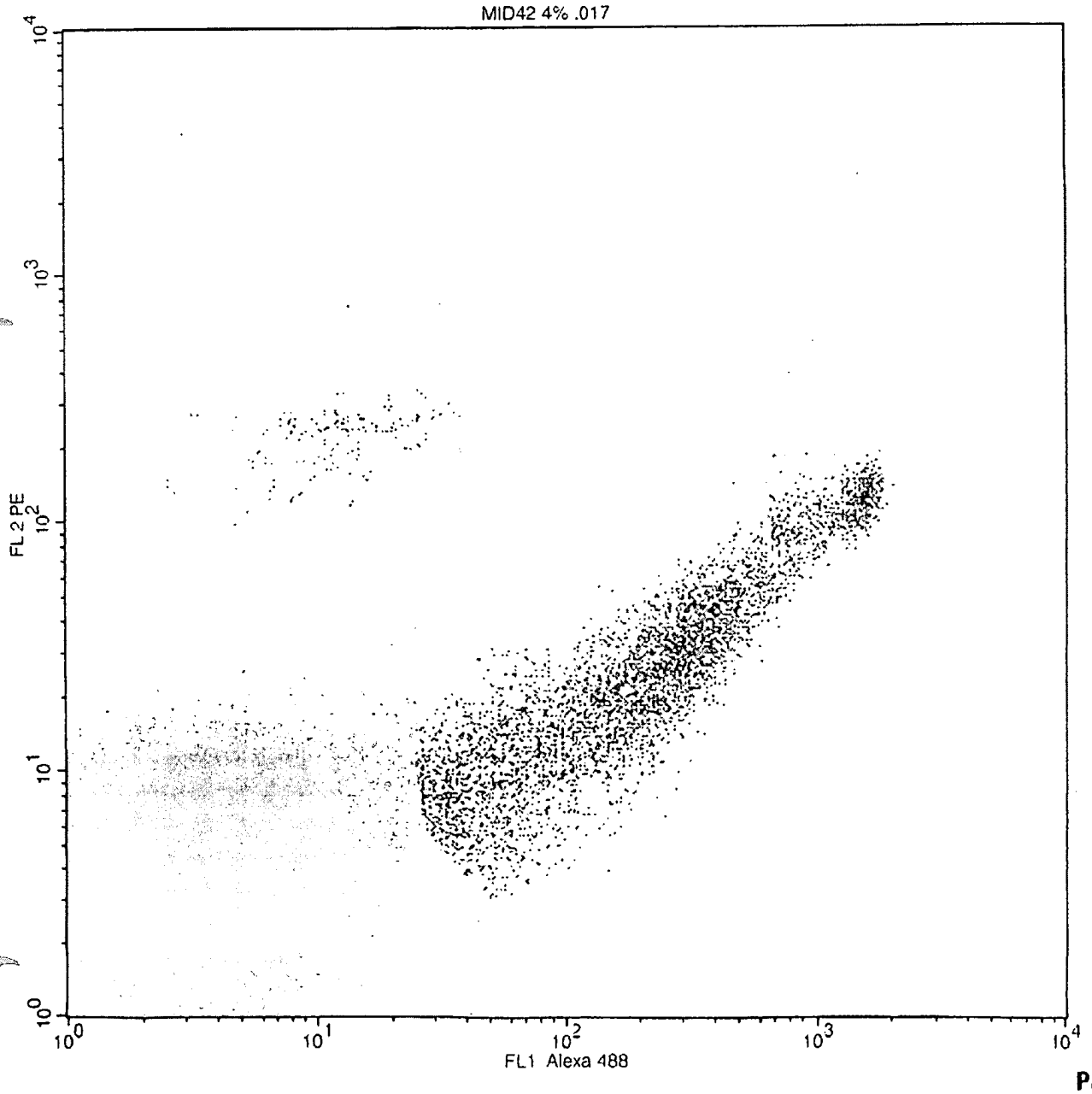
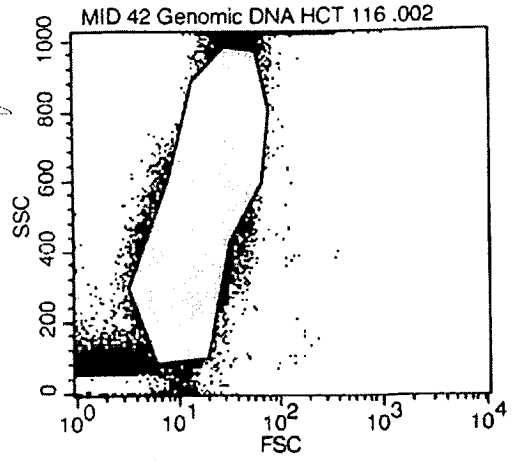


Exhibit L



File: MID 42 Genomic DNA HCT 116 .00:
Acquisition Date:
Total Events: 338790

Region	Events	% Gated
R1	320996	100.00
Green Gate	1087	0.34
Red Gate	1040	0.32
R4	3336	1.04

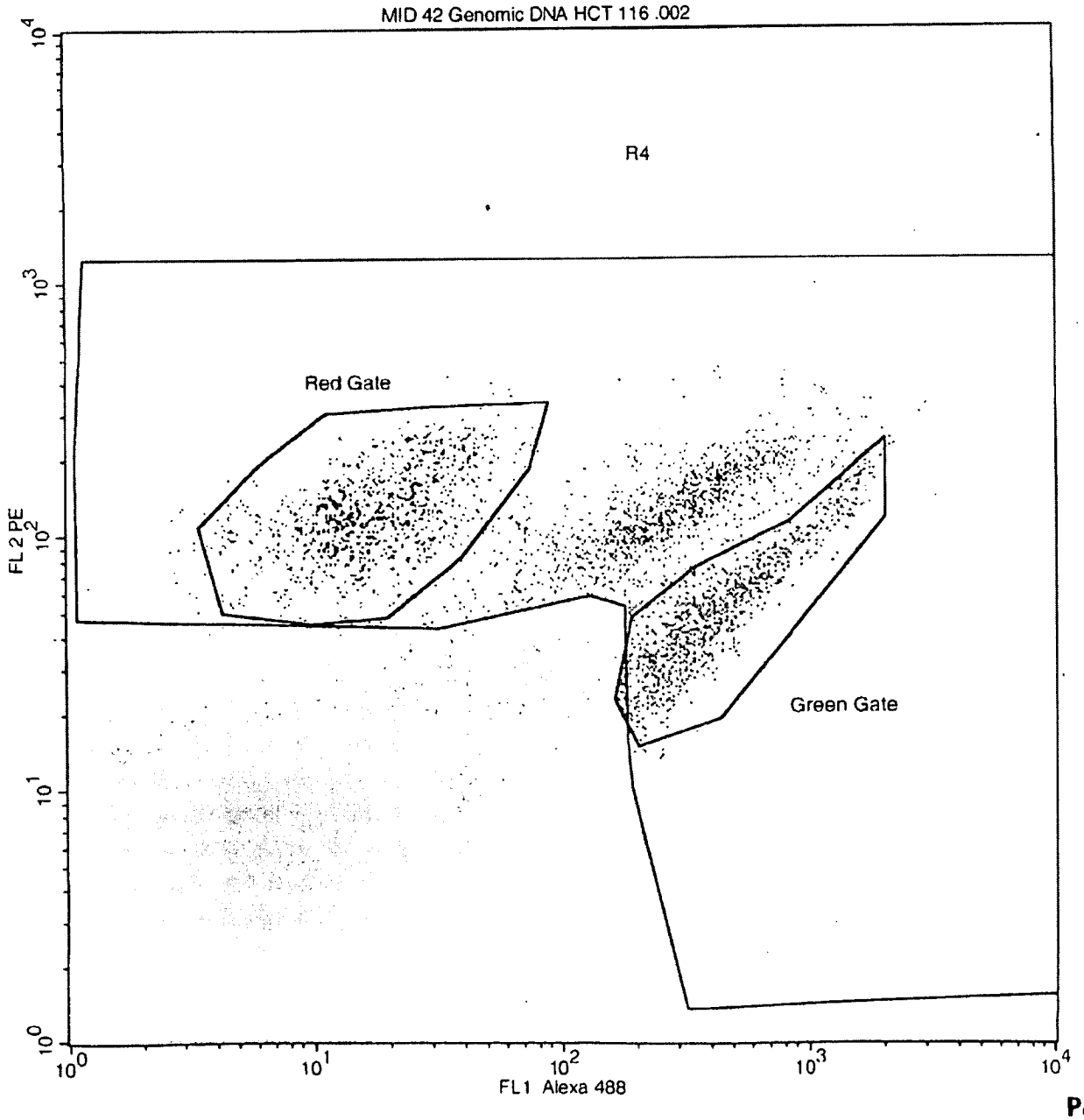
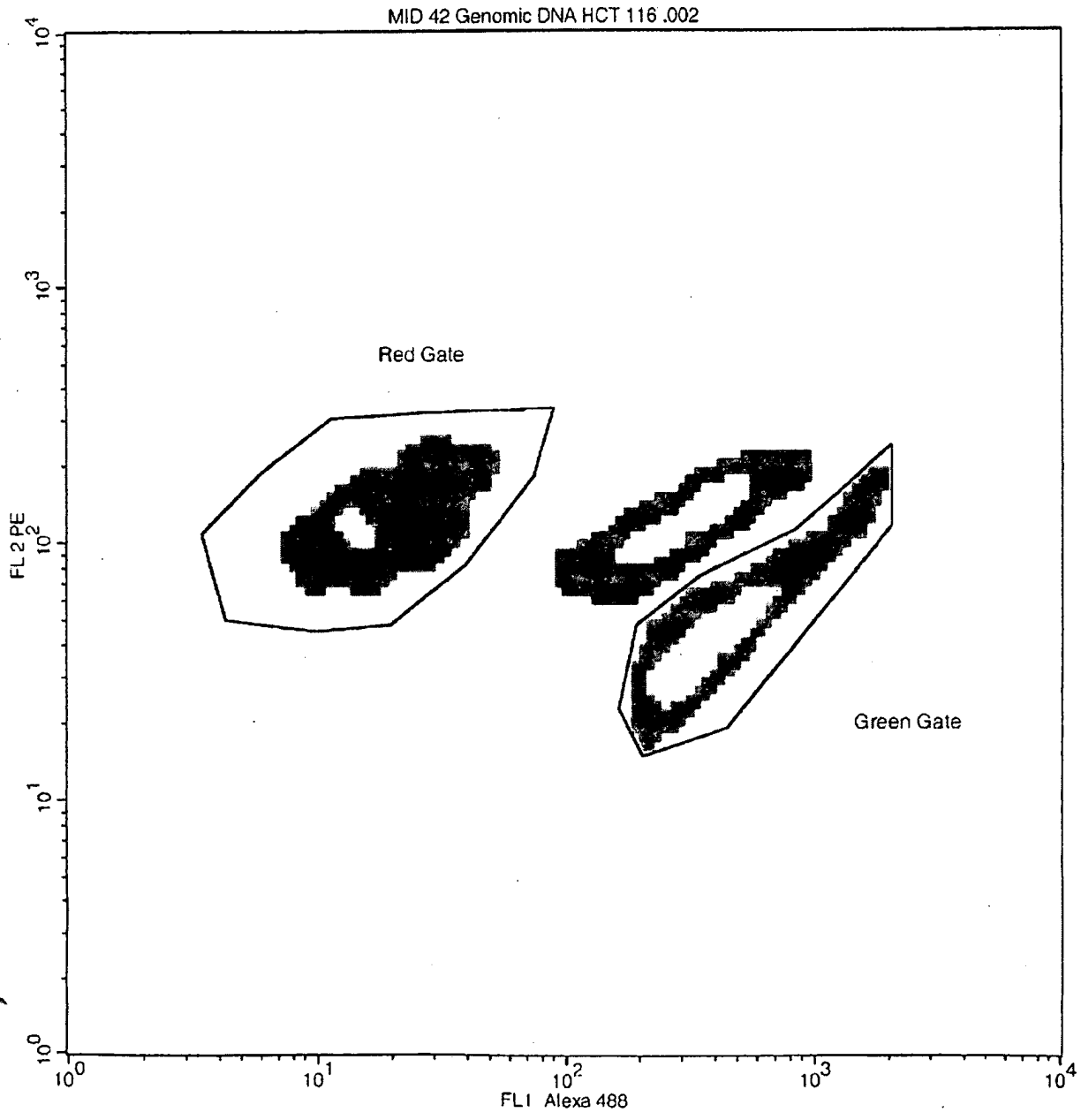


Exhibit M


MID 42 Genomic DNA HCT 116 .002
Red Gate
Green Gate
FL 2 PE
FL1 Alexa 488
10^0
10^1
10^2
10^3
10^4